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(54) Title: CONTRACEPTIVE VACCINE BASED ON ALLOIMMUNIZATION WITH ZONA PELLUCIDA POLYPEPTIDES

(57) Abstract

The present invention relates to contraceptive vaccines based on cloned zona pellucida genes and the strategy of alloimmunization with zona pellucida polypeptides. In particular, the present invention relates to a contraceptive vaccine for use in a mammalian female comprising a polypeptide which displays at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm. This epitope is from a zona pellucida protein of the species in which the said vaccine is used. This invention relates, more particularly, to such vaccines wherein the zona pellucida protein is either the ZP3 or the ZP2 or the ZP1 protein of the mouse or homologues of these proteins in some other mammalian species. Further, this invention comprehends vaccines comprising a synthetic peptide that displays an epitope for such an antibody that inhibits fertilization. In addition, this invention relates to cloned DNA segments variously encoding the mouse ZP3 or ZP2 proteins or the human ZP3 or ZP2 protein.

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**CONTRACEPTIVE VACCINE BASED ON
ALLOIMMUNIZATION WITH ZONA PELLUCIDA POLYPEPTIDES**

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Background of the Invention

The present invention relates to contraceptive vaccines based on cloned zona pellucida genes and the strategy of alloimmunization with zona pellucida polypeptides. In particular, the present invention relates to a contraceptive vaccine for use in a mammalian female comprising a polypeptide which displays at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm. This epitope is from a zona pellucida protein of the species in which the said vaccine is used.

This invention relates, more particularly, to such vaccines wherein the zona pellucida protein is either the mouse ZP2 protein, the mouse ZP3 protein, the human ZP2 protein, the human ZP3 protein, or homologues of these proteins found in other mammalian species. Further, this invention includes vaccines comprising a synthetic peptide that displays an epitope for such an antibody that inhibits fertilization. In addition, this invention relates to cloned DNA segments variously encoding the mouse ZP3 or ZP2 proteins, or the human ZP2 or ZP3 proteins.

There is currently much interest in the development of a safe and effective contraceptive vaccine for control of diverse mammalian populations. Contraceptive vaccines would be useful under certain circumstances where relatively long-term but not permanent contraception is desired without the need for frequent intervention, for example, in pets including cats and dogs, in agriculturally important livestock such as cattle and pigs, and in human beings. A contraceptive vaccine preferably should have an effect which is long-lasting and highly specific. Further, to minimize possibilities for birth defects in the event of failed contraception, the antigen which is selected as the immunogen should produce contraceptive antibodies that inhibit fertilization of the egg by a sperm rather than by an abortifacient mechanism involving disruption of early development. In addition, the vaccine

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preferably should induce an immunological response that is sufficient to be effective for contraception without eliciting a cytotoxic response that might result in abnormal reproductive function.

5 The mammalian zona pellucida, which surrounds growing oocytes and ovulated eggs, has been recognized as a potential immunogen for a contraceptive vaccine (C.J. Henderson, et al., J. Reprod. Fert. 83: 325-343 (1988); B.S. Dunbar, 1983, Mechanisms and Control of Animal
10 Fertilization, J.F. Hartmann, ed., pp. 140-175, Academic Press, New York; A.T. Sacco, Am. J. Reprod. Immunol. Microbiol. 15: 122 (1987); Millar et al., Targeting of zona pellucida for immunocontraception, in Immunology of Reproduction, Naz, R.K. (ed.), pp. 293-313 (1993)). At
15 birth the mouse ovary contains 10,000-15,000 oocytes in the prophase of the first meiotic division. As cohorts (10-15) of these oocytes enter into a two week growth phase, they synthesize and secrete zona proteins to form the extra-cellular zona pellucida which ultimately reaches
20 a thickness of 7 μ m in the fully grown oocyte. The zona is unique to the ovary, being highly antigenic and accessible to circulating antibody during the two week intra-ovarian oocyte growth phase prior to meiotic maturation and ovulation.

25 Passive immunization of mice or hamsters with anti-zona sera has been shown to produce reversible contraception without obvious side effects. For example, U.S. Patent 3,992,520 to Gwatkin discloses, *inter alia*, an anti-serum composition for short-term control of fertility
30 comprising antibody obtained by immunizing an animal with water solubilized zona pellucida of a distinct donor species. This method requires isolation of large amounts of a relatively scarce natural antigen which would not be feasible for certain mammals such as humans. Further,
35 long-term administration of antibodies from a foreign (i.e., "heterologous") species leads to induction of reactive antibodies that will inhibit the contraceptive action of the contraceptive antibodies. Further, administration of serum or products isolated from serum carries

inherent risks of transmission of blood-born diseases.

Structural information about the zona pellucida has been available for some years. The mouse zona, for instance, is composed of three sulfated glycoproteins, 5 designated ZP1, ZP2 and ZP3, (J.D. Bleil et al., Dev. Biol. 76:185 (1980); S. Shimizu et al., J. Biol. Chem. 258:5858 (1983)) which play important roles in fertilization and early development and have average M_r 's of 200,000, 140,000, and 85,000, respectively. ZP2 and ZP3 appear to 10 be complexed into long filaments which are cross-linked by ZP1 in the zona matrix providing structural integrity to the zona pellucida. Sperm initially bind to ZP3 via O-linked oligosaccharide chains and continued binding involves ZP2 as a secondary sperm receptor. Subsequently, 15 ZP3 induces lysis of the sperm's acrosome which releases enzymes (such as glycosidases and proteases) which are thought to be important for the penetration of the zona pellucida by sperm. Following fertilization, both ZP2 and ZP3 are biochemically modified to prevent additional sperm 20 binding and thereby to facilitate the post-fertilization block to polyspermy.

The zona pellucida in other mammals besides the mouse is known to comprise several distinct glycoproteins components with apparent sizes and, hence naming terminologies, that do not necessarily correspond directly to the 25 mouse ZP1 (185-200 kDa), ZP2 (120-140 kDa) and ZP3 (83 kDa) proteins. The human zona pellucida is composed of three proteins designated ZP1 (90-110 kDa), ZP2 (64-76 kDa) and ZP3 (57-73 kDa) (Shabanowitz et al., J. Reprod. Fertil. 82:151-61 (1988); Shabanowitz, 43:260-70 (1990)) and other species in which zona proteins have been characterized include hamster (Moller et al., 137:276-86 (1990)), pig (Dunbar et al., Biol. Reprod. (1981); Hedrick et al., Dev. Biol. 121:478-88 (1987); Yurewicz et al., J. Biol. Chem. 262:564-71 (1987)), rabbit (Dunbar et al., Biol. Reprod. 24:1111-24 (1981) and horse (Millar et al., J. Reprod. Fert. 96:815-25 (1992)). The correspondence of 30 specific zona proteins among different species is becoming clearer as additional information on the primary amino 35 acid sequence of the proteins is obtained.

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acid sequence is deduced from cloned zona pellucida genes (Ringuette et al., Proc. Natl. Acad. Sci. U.S.A. 83:4341-45 (1986); Ringuette et al., Dev. Biol. 127:287-95 (1988); Chamberlin et al., Proc. Natl. Acad. Sci. U.S.A. 87:6014-18 (1990); Chamberlin et al., Dev. Biol. 131:207-14 (1989); Liang et al., Mol. Cell. Biol. 10:1507-15 (1990); Liang et al., Dev. Biol. 156:399-408 (1993); Kinloch et al., Dev. Biol. 142:414-21 (1988); Schwoebel et al., J. Biol. Chem. 266:7214-19 (1991); Kinloch et al., Dev. Biol. 142:414-21 (1990)) and direct sequencing of peptides derived from zona pellucida proteins (Ringuette et al., supra (1986); Yurewicz et al., Mol. Reprod. Dev. 33:182-88 (1992)).

In light of the identification of the distinct murine zona pellucida polypeptides, ZP1, ZP2 and ZP3, further experiments on passive immunization with contraceptive antibodies have been conducted. Specifically, rat anti-mouse ZP2 and anti-mouse ZP3 monoclonal antibodies were injected into female mice and were found to bind specifically to the zonae surrounding growing, intra-ovarian oocytes. After ovulation, the binding of the antibody to the zona persisted; and the presence of these antibodies precluded fertilization by preventing sperm from penetration of the zona pellucida. This contraceptive effect was long-term, lasting approximately 15 mouse estrus cycles, but was eventually reversible. There was no evidence of any adverse effect on the development of fertilized embryos to term and no evidence of abnormal ovarian histology or function. However, the antibody binding sites (i.e., "epitopes") recognized on mouse ZP2 and ZP3 by five different rat anti-mouse monoclonal antibodies that were tested are not present on other mammalian zonae pellucidae (East et al., J. Cell Biol. 98:795-800 (1984); East et al., Dev. Biol. 104:49-56 (1984); and East et al., Dev. Biol. 109: 268-73 (1985)). This species specificity limits the usefulness of these particular antibodies as contraceptive agents essentially to murine species. In addition, even if analogous murine anti-ZP2 or anti-ZP3 antibodies that inhibit fertilization could be

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identified for ZP2 or ZP3 of non-murine species, there are inherent side-effects from the repeated administration of heterologous antibodies, as noted above.

There have been several studies on active 5 immunization using preparations of isolated zona pellucidae to immunize rodents, rabbits, and primates (C.J. Henderson, et al., J. Reprod. Fert. 83:325 (1988); R. B. L. Gwatkin, et al., 1977, Fert. Steril. 28:871 (1977); Drell et al., Biol. Reprod. 30:435-44 (1984); Sacco et 10 al., Biol. Reprod. 36:481-90 (1987); Jones et al., J. Reprod. Fertil. 95:513-25 (1992)).

Further, the U.S. Patent to Gwatkin cited above (U.S. 3,992,520) also discloses a vaccine for the immunological control of fertility in female mammals that 15 consists of an aqueous solution of water solubilized zona pellucida prepared by heating mammalian zone pellucida at 65-100°C in an aqueous medium. One example therein describes a bovine antigen preparation intended for use in humans.

20 U.S. Patent 4,996,297 of Dunbar is limited to three rabbit cDNA sequences S1, P2, and P3 thought to encode rabbit zona proteins, to the use of these cDNAs to produce polypeptides that contain epitopes on three rabbit zona proteins (50 kDa, 75kDa, and 80 kDa), and to the use 25 of the recombinant polypeptides to vaccinate other mammals in order to elicit antibodies that bind to that mammal's zona pellucida for contraception (i.e., heteroimmunization).

Japanese Patent 63,150,299 discloses a pig zona 30 pellucida antigen for use as a contraceptive vaccine for pigs or humans that is characterized as a glycoprotein of 20 to 30 kDa in molecular weight which can be extracted from soluble pig zona pellucida with 8.5 M urea and 2% 2-mercaptoethanol.

35 Despite positive results under experimental conditions, methods of preparing a vaccine from natural zona pellucida materials are clearly difficult if not outright impractical for commercial use, particularly in the human case, due to limited sources of antigen and to

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difficulties in quality control of such poorly defined vaccines. Further, wide-spread ovarian histopathology and dysfunction were reported in rabbits, dogs and primates after active immunization with zonae pellucidae or extracted antigens (see, for example, R.B.L. Gwatkin, et al., Gamete Res. 1:19 (1980); A.T. Sacco, Am. J. Reprod. Immunol. Microbiol. 15:122 (1977)). Several studies have suggested that both the dose and the purity of the immunogen contributed to these abnormalities, two properties that are particularly difficult to control in such relatively crude antigen preparations.

The effect of the genetic origin of the zona pellucida antigen on its ability to immunize a given species against conception has been examined in several studies. For instance, the efficacies of contraceptive immunizations with pig and rabbit zonae pellucidae on fertility in rabbits was compared. This comparison of results with "alloimmunization" (literally "self-immunization", using antigen from the same species, i.e., an "alloantigen") with those of "heteroimmunization" (using antigen from another species, i.e., an "heterologous" antigen) suggested (D. M. Wood et al., Biol. Reprod. 25:439-450 (1981)) that heteroimmunization of rabbits with porcine zonae is more effective in reducing fertility than alloimmunization with rabbit zonae. More recent work using immunoaffinity purified antibodies to zona pellucida to compare immune responses in alloimmunization of male and female rabbits has continued to support the greater effectiveness for contraception of heteroimmunization with zona pellucida antigens. (S. M. Skinner, et al., J. Reproductive Immunology 12:81-92 (1987)).

Another general approach toward providing a vaccine related to any antigen involves the use of a particular type of antibody, called an "anti-idiotypic" antibody, as an immunogen to actively immunize an animal. Anti-idiotypic antibodies are antibodies directed to the antigen binding site of another antibody; accordingly, the antigen binding site of the anti-idiotypic antibody mimics or represents an image of the site on the antigen that is

bound by the other antibody. U.S. Patent 4,795,634 to Grimes et al. (equivalent of WO 87/05,516) discloses a vaccine that comprises anti-idiotypic antibodies to anti-zona pellucida antibodies to express images of zona pellucida antigens. This vaccine suffers from drawbacks including the fact that anti-idiotypic antibodies are generally difficult and expensive to prepare in amounts and purity satisfactory for vaccine usage, particularly in human applications. Further, heteroimmunization with antigens comprising antibodies from another species may induce predominantly antibodies to sites on the antibody other than the desired target, the antigen binding site. In other words, the desired antigen binding site may not constitute an "immunodominant" antigenic site (or "determinant") for the vaccine antibody protein in a species different from that which produced the vaccine protein (see below for a discussion on the basis of immunodominance). (See also U.S. Patent 4,996,297 of Dunbar et al.)

Another technique for producing vaccines that is known generally in the art is the use of specific isolated polypeptides as antigens, or of peptides representing portions of such polypeptides, in place of crude antigen preparations comprising aqueous extracts of target tissues. Accordingly, European Patent EP-0117934 to Stevens discloses a modified antigen for use in fertility control comprising an unspecified antigen from the zona pellucida, or a peptide having a sequence corresponding to at least part of the sequence of such a zona pellucida antigen, which antigen or peptide has been chemically modified outside the body of the animal. The modified antigen has a greater capacity to induce antibodies than the unmodified antigen from which it is derived. According to the specification and claims, such modification includes coupling the antigen or peptide through a maleimido linkage to a suitable "carrier" protein that is biologically foreign to the animal to be vaccinated and of size sufficient to elicit antibody response. Neither this European application nor any related applications, as yet published, teaches specific zona pellucida polypeptides or

peptides that are suitable for use as contraceptive vaccines.

In light of the complexities, difficulties and uncertainties of all the contraceptive vaccines described 5 above, there is yet a need for a simpler, safer, cheaper, more defined and effective contraceptive vaccine. The present invention is based on the premise that vaccination with a "self" zona protein (alloimmunization) is most likely to elicit antibodies that will cross-react with the 10 native zona pellucida and prevent fertilization. Furthermore, by using relatively short peptides as immunogens, the adverse effects on ovarian structure and functions, at least some of which can result from a T cell mediated 15 autoimmune response, can be avoided. However, the success of this approach depends on knowledge of the primary amino acid sequence of the zona pellucida proteins. Because of the paucity of biological material, this sequence information can only be obtained by cloning cDNAs encoding the zona proteins and deducing the amino acid sequence from 20 the nucleic acid sequence. Toward this end, the present inventor and associates have recently constructed a mouse ovarian cDNA expression library and isolated two overlapping ZP3 cDNA clones (M. J. Ringuette et al., Proc. Natl. Acad. Sci. USA 83:4341 (1986)), one of which expresses a 25 fusion protein recognized by an anti-ZP3 monoclonal antibody (East et al., Dev. Biol. 109: 268 (1985)).

The identity of these clones was confirmed by a comparison of the amino acid sequence encoded by a 60 nucleotide stretch of their nucleic acid sequence with the 30 terminal amino acid sequence (20 amino acids) of a large internal fragment isolated from the ZP3 protein (Ringuette et al., supra 1986)). This fragment was isolated from purified ZP3, following digestion with a protease, by affinity chromatography using an anti-ZP3 monoclonal 35 antibody. Therefore, it was clear that this fragment was capable of expressing an epitope for a contraceptive antibody; however, the location of that epitope within scores of amino acid residues was not known, and as disclosed herein, is distinct from the 20 amino acid

sequence obtained. More importantly, the ability of this proteolytic cleavage fragment to serve as an immunogen in a vaccine was not known, nor was there any practical means for preparing sufficient material from natural sources to 5 test that cleavage fragment further.

A first attempt to utilize the cloned mouse ZP3 cDNA described above to produce a vaccine was unsuccessful (S. M. Chamow and J. Dean, 1987, abstract of presentation to the American Society of Biological Chemists). This 10 effort involved testing of the recombinant ZP3- β -galactosidase fusion protein, which contained most of the ZP3 amino acids as well as a larger portion of β -galactosidase and was generated according to well known methods in genetic engineering that have successfully produced other 15 antigens with native immunoreactivity. Immunization with this particular fusion protein, however, failed to induce detectable antibodies that would react with native ZP3; reactivity was detected only after reduction of disulfide bonds and denaturation.

20 The basis of this failure to induce anti-ZP3 contraceptive antibodies, despite that fact that the cDNA clearly encoded a proteolytic cleavage fragment that reacted with such an antibody, is not entirely clear. It may be that, under the conditions of immunization, the 25 portion of the fusion protein that encoded the contraceptive antibody epitope did not assume the proper conformation to react with such antibodies. In other words, although the fusion protein surely encoded the amino acids that formed the epitope in the native ZP3 protein, it may 30 be that those amino acids did not exhibit (i.e., did not "display") that epitope in this instance. It is also possible that epitopes for other antibodies, which were located on the β -galactosidase moiety of the fusion, may 35 have been immunodominant over the contraceptive antibody epitopes and thus prevented a detectable contraceptive antibody response (see discussion of immunodominance below). Finally, a combination of these effects and others may have united to prevent the desired contraceptive antibody response to the fusion product of the

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recombinant DNA which expressed most of the ZP3 polypeptide. These results clearly illustrate the unpredictability of the immunogenicity of a polypeptide under any given set of conditions, no matter how efficacious they may be 5 for other antigens, and the need for experimental determination of the necessary physical form of the amino acids that encode an epitope (e.g., polypeptide size and nature of attached amino acid sequences) to display that epitope and, further, to induce antibodies to it.

10 Accordingly, it is an object of the present invention to find an efficacious way to use contraceptive antibodies and cloned genes encoding zona pellucida proteins to develop contraceptive vaccines for use in a mammalian female. More particularly, it is an object of 15 this invention to provide such vaccines comprising polypeptides that include defined amino acid sequences that are selected for their ability to display epitopes for contraceptive antibodies.

20 Additional immunological analyses of the individual ZP polypeptide components have been carried out. For example, specific monoclonal and polyclonal antibodies have been employed to define distinct antigens of the porcine zonae pellucidae, leading to the suggestion that there are both unique and shared antigenic determinants 25 present in the individual components of the zona pellucida, but that the immunodominant determinants appear to be unique to each glycoprotein (T. M. Timmons, et al., Biology of Reproduction 36:1275-1287 (1987)).

Finally, there has been a report of an effort to 30 molecularly clone cDNAs encoding specific antigenic sites from rabbit ZP proteins using antibodies that recognize determinants found on ZP antigens of several species (P. Cheung et al., 1987, abstract of a presentation at the twenty-seventh annual meeting of the American Society for 35 Cell Biology, St. Louis, Missouri, November 16-20, J. Cell Biol. 105, no. 4 part 2, 334A). This abstract reported in part that:

5 "These studies demonstrated that cross-species affinity purification of antibodies is an effective method for isolating cDNA clones expressing antigens which are shared among different mammalian species."

However, no specific nucleotide or amino acid sequences were disclosed in this abstract, nor was the contraceptive potential of the antibodies discussed; indeed, there was 10 no mention of any contraceptive vaccine.

In a speculative exposition on the use of recombinant DNA and synthetic peptide technologies for development of a human contraceptive vaccine from porcine zona pellucida antigens (C.J. Henderson, et al., J. Reprod. Fert. 83:325 (1988)), which was entitled "The future . . .", the identification of amino acid sequences displaying epitopes for contraceptive vaccines on a particular porcine polypeptide is anticipated, although absolutely no sequences of the polypeptide are disclosed.

Nevertheless, this reference goes on to hypothesize that known vaccine technologies, including synthetic peptides and vaccinia virus expression vectors, will provide successful human vaccines based on this particular porcine polypeptide that is known to be immunologically related to human zona pellucida antigens. Furthermore, while asserting that monoclonal antibodies to this polypeptide that exert a contraceptive effect "will be extremely important in defining the epitopes with contraceptive potential . . .", this report also notes that, despite obtaining monoclonal antibodies reactive with this polypeptide, the authors "have failed to generate a monoclonal antibody with contraceptive effect; this is in accord with other published reports . . ."

35 Although a complete exposition of the current theoretical basis of immunogenicity and antigenicity of polypeptides is beyond the scope of the present disclosure, a brief discussion of selected principles and terms of this active art will facilitate further understanding of the instant invention. [In this application, absent an

express statement to the contrary, each use of the term "polypeptide" encompasses any polymer comprising two or more amino acids coupled by peptide linkages (i.e., dipeptides, oligopeptides, peptides, polypeptides) as well 5 as proteins consisting of multiple polypeptide subunits.]

Accordingly, it should be noted, first, that the necessary and sufficient properties of a polypeptide for inducing antibodies cannot be predicted for any given set of conditions (e.g., for a particular species, or for 10 presentation in a certain form). Nevertheless, much more has been learned about this subject in the past decade than is reflected in any of the art cited so far herein, and it is a further object of the present invention to 15 exploit aspects of this knowledge for design of advantageous contraceptive vaccines.

In particular, comprehension of the present invention will be aided by the now widely held view that the nature and level of the immune response to a polypeptide depends on its interactions with at least two distinct 20 classes of immune system cells, namely B-cells and T-cells. In simple terms, the role of B-cells in immunity may be thought of as recognition of the specific sites on macromolecules to which antibodies are produced and subsequent production of those antibodies. These B-cell 25 recognition sites, which provide the main basis for immune recognition of non-self molecules and are also called B-cell epitopes, are of a size corresponding to about that of the antigen binding site on an antibody, typically of a diameter equivalent to the length of a peptide containing 30 about four to six amino acids.

[It may be noted here that there exists a formal distinction between the epitope for a B-cell and that of its related antibody. In other words, due to complex biological mechanisms that intervene between the recognition 35 by a B-cell of a given site on an antigen and the consequent production of antibodies to that site, it is possible that the ultimate antibody recognition site may not be precisely identical to the initially recognized B-cell epitope. However, for the present purposes, a B-cell

epitope may be considered to be essentially the same structure as the binding site for the corresponding antibody.]

The functions of T-cells, on the other hand, 5 relate in large measure to helping to activate antibody production by B-cells upon initial exposure to an antigen, as well as to enhancing their antibody response upon subsequent reexposures (i.e., to "immune memory" or the "amnestic" response). To play their roles in immunity, T- 10 cells must also recognize specific sites on an antigen to which antibodies are produced, and such T-cell epitopes are about the same size as B-cell epitopes.

B-cell and T-cell epitopes on any given polypeptide, however, need not comprise the same amino acid 15 residues. In fact, it will be appreciated by those of ordinary knowledge in the current art of peptide immunology at the molecular level, that even in a peptide consisting of only half a dozen amino acids, there may coexist several different B-cell epitopes (comprising, for in- 20 stance, from two to four atoms that contact complementary structures on the antibody) and one or more distinct T-cell epitopes which may or may not include atoms of amino acids also included in a B-cell epitope.

It is also well known that the vast majority of 25 small peptides (containing six to twenty amino acids, for instance) that have been tested for induction of antibodies are considerably less potent immunogens than the larger proteins from which they have been derived, despite ample ability of the peptides to bind to antibodies 30 directed against those larger proteins. Certain chemical modifications of a peptide, particularly coupling of the peptide to a larger proteinaceous "carrier", generally enhance the immune response to a small peptide.

Although the role of such a carrier still may 35 not be fully understood in all respects, it has been clearly established, in particular, that there is no specific minimum size requirement for peptides in general to induce a substantial immune response. Rather, it is now widely believed that a major function of the carrier

is to provide T-cell epitopes in close association with the B-cell epitopes on the short peptide which is statistically unlikely to contain both T-cell and B-cell sites recognized by the immune system of any given individual.

5 It may also be noted here that it has been shown that a T-cell epitope taken from one protein, in the form of a short peptide, may be combined with a short peptide comprising a B-cell epitope of another protein, to form a single peptide that induces a more complete and higher
10 level immune response than either peptide alone.

More broadly, it is now widely accepted that the capability of any individual to mount any immune response to a given epitope, as defined by a precise configuration of a small number of atoms, depends ultimately on the
15 genetic make-up of the immune system genes which separately control the specificities of antigen recognition by B-cells and T-cells. Further, it is understood that the ability of a given B-cell epitope to induce cognate antibodies (i.e., antibodies which recognize that epitope)
20 also depends upon the context within which that epitope is presented to the immune system, in terms of both associated T-cell epitopes and other B-cell epitopes. The latter sites may be "immunodominant" relative to the selected B-cell epitope of interest, that is, they may contend more
25 effectively for the attention of the immune system than the selected B-cell epitope and thereby distract limited system resources from mounting the desired response to that selected epitope. In other words, B-cell epitopes that do not induce detectable antibodies in the presence
30 of other, so-called immunodominant epitopes, which frequently occur in large polypeptides, often do induce significant levels of cognate antibodies when presented in a different context that lacks such immunodominant sites, on a short peptide, for example.

35 In conclusion, it is a further object of the present invention to exploit various consequences of the above noted characteristics of and distinctions between B-cell and T-cell epitopes, as well as methods for predicting and actually detecting amino acid sequences that serve

as T-cell or B-cell epitopes. These will be discussed further below, as needed, in relation to the description of the present invention.

5 Summary of the Invention

The recent molecular cloning, by the present inventor, of DNA segments corresponding to the mouse ZP3 and ZP2 genes, the human ZP3 and ZP2 genes, and the subsequent characterization of the nucleotide sequences of 10 their messenger RNAs (mRNAs) and the amino acid sequences encoded thereby, have provided sufficient molecular detail of zona proteins to enable a new contraceptive approach. This strategy is based on active alloimmunization with a zona pellucida polypeptide which includes an amino acid 15 sequence that is selected to display at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm.

The complete nucleotide sequence of the mouse ZP3 messenger RNA and the amino acid sequence encoded 20 thereby has been disclosed previously by the present inventor and associates (M.J. Ringuette et al., Dev. Biol. 127:287-296 (1988), published June 13, 1988, the entire contents of which are hereby incorporated herein by reference). The complete nucleotide sequence of the mouse 25 ZP2 (Liang et al., Mol. Cell. Biol. 10:1507-15 (1990)), the human ZP3 (Chamberlin et al., Proc. Natl. Acad. Sci. U.S.A. 87:6014-18 (1990)) and the human ZP2 (Liang et al., Mol. Cell. Biol. 10:1507-15 (1993)) messenger RNAs and the amino acid sequences encoded thereby have also been 30 disclosed and the entire contents of the published documents are hereby incorporated herein by reference.

The present inventor and associates have also reported (M. Chamberlin et al., 1987, abstract of a presentation at the twenty-seventh annual meeting of the 35 American Society for Cell Biology, St. Louis, Missouri, November 16-20, J. Cell Biol. 105, no. 4 part 2, 334A) that mouse genomic clones of the ZP3 gene and a human genomic DNA clone of the ZP3 gene have been isolated by virtue of their homology to the previously isolated murine

ZP3 cDNAs. However, this abstract does not disclose specific nucleotide or amino acid sequences of any mouse or human DNA clone, nor does it even mention any concept of a contraceptive vaccine. Further, the mouse and human 5 ZP2 cDNA sequences have not been disclosed previously.

Enabled by an oligonucleotide probe based on the short ZP3 cDNA sequence that was published by the present inventor and associates (Ringuette et al., supra (1986)), and subsequent to publication of the complete mouse ZP3 10 cDNA sequence (M.J. Ringuette et al., Dev. Biol. 127:287 (1988)), others have also reported isolation and sequences of genomic DNA clones of a mouse ZP3 gene and the amino acid sequence encoded therein (R. A. Kinloch et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:6409-413 (1988)). This 15 information was also used to isolate genomic DNA clones of hamster ZP3 and, by comparison with previously described mouse ZP3 genes, to deduce the amino acid sequence of the resultant polypeptide chain (Kinloch et al., Devel. Biol. 142:414-21 (1990)). Independently, others have reported 20 the isolation of a cDNA encoding rc55, a rabbit zona pellucida protein (Schwoebel et al., J. Biol. Chem. 266:7214-19 (1991)), that does not appear to be the homologue of either mouse ZP2 or ZP3.

Whereas the prior art on contraceptive vaccines 25 based on zona pellucida antigens has been and remains primarily focused on heteroimmunization, the present invention relates to contraceptive vaccines based on cloned zona pellucida genes and the strategy of alloimmunization with polypeptides including defined amino 30 acid sequences that are selected for displaying epitopes to contraceptive antibodies. The advantages of this approach include the ability to produce and utilize those immunogens displaying the most effective B-cell epitopes for inhibition of fertilization regardless of whether or 35 not they happen to be conserved in several species. Further, this vaccine strategy minimizes the likelihood of inducing antibodies with deleterious cross-reactivity with epitopes on molecules other than zona pellucida polypeptides. Ultimately, by reducing in the vaccine the

number of B-cell epitopes that produce antibodies which, even though they bind to a zona pellucida antigen, do not block conception, this invention focuses the immune response to the vaccine on precisely those amino acids 5 that are most critically situated to facilitate the contraceptive effect of antibodies. Further, by focusing on those epitopes that are most useful for contraceptive purposes, the present invention minimizes potential interference with establishment of effective immunity to 10 those critical contraceptive epitopes from extraneous epitopes that may be immunodominant to those critical sites and, therefore, may prevent an adequate contraceptive antibody response to them. In addition, by focusing on these epitopes, the potential for adverse immunological 15 response due to the induction of autoimmune responses can be minimized (Rhim et al., J. Clin. Invest. 89:28-35 (1992)).

It is understood that, in the practice of the present invention, epitopes may be used which happen to be 20 conserved in the zona pellucida proteins of more than one species. However, in contrast to previous efforts to employ zona pellucida antigens in vaccines wherein the first concern has been to identify cross-reacting epitopes 25 in heterologous antigens without initial regard for the functionality of such epitopes in inducing contraceptive antibodies, as described in some references cited herein above, it will be appreciated that use of conserved epitopes in the instant invention is entirely incidental to the goal of providing epitopes that are effective for 30 inducing contraceptive antibodies in the particular target species intended for a given vaccine.

Accordingly, the present invention relates to a contraceptive vaccine for use in a mammalian female comprising a polypeptide which includes an amino acid 35 sequence that is selected to display at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm. This contraceptive antibody epitope is an epitope for which there is a functional homolog displayed on a zona pellucida protein that originates from

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the species in which the said vaccine is used. The zona pellucida protein displaying the functionally homologous epitope advantageously is either a ZP3 protein or a ZP2 protein or a ZP1 protein.

5 In other words, both the amino acid sequence of a polypeptide of this vaccine and a zona pellucida protein display epitopes which are functionally homologous in that they both are able to bind the same antibody that inhibits fertilization of an oocyte by a sperm. The fact that this
10 vaccine polypeptide and a zona pellucida protein both display functionally homologous binding sites for the same antibody does not imply, however, that these binding sites are encoded by the same amino acid sequence in each instance, i.e., the polypeptides displaying the two
15 epitopes are not necessarily structurally homologous at the level of amino acid sequences encoding the epitopes.

By the phrase "originating from" it is meant that the zona pellucida protein is encoded in the genome of the species in which the said vaccine is used.

20 It will be understood from the foregoing Background that the nomenclature of zona pellucida proteins comprising ZP1, ZP2 and ZP3 has been defined in the mouse system and that other nomenclature or no nomenclature may be used in other mammalian systems. However, the present
25 inventor has clearly demonstrated that the genes and mRNAs and, hence, the amino acid sequences of the major murine zona pellucida proteins (for example, the ZP3 and ZP2 proteins of the mouse) are conserved throughout diverse mammalian species (see below). In light of this high
30 degree of structural similarity, a high degree of functional homology is also to be expected in terms of the ability of homologous positions to serve as epitopes of contraceptive antibodies. Accordingly, the terms "ZP3 protein", "ZP2 protein", and "ZP1 protein" contemplate not
35 only the murine forms of these highly conserved zona pellucida proteins, but also the homologous counterparts of any other mammalian species, regardless of any other terminology by which such other proteins may be known in the art.

Contraceptive antibodies suitable for the practice of the present invention may be generated using zona pellucida antigens from natural sources, according to various published procedures. Alternatively, such antibodies 5 may be produced advantageously by immunization with a polypeptide produced in a recombinant expression system comprising a DNA segment of the present invention. Various methods for identifying antibodies, including monoclonal antibodies, that inhibit the fertilization of 10 an oocyte by a sperm have also been published (e.g., East et al., Dev. Biol. 109:268 (1985)).

In the polypeptide of the vaccine of this invention, the amino acid sequence which displays an epitope for a contraceptive antibody may include all or 15 part of the same amino acid sequence responsible for displaying the functionally identical epitope on a zona pellucida protein. In some cases, a single epitope for binding a given antibody comprises more than one contiguous amino acid sequence of a polypeptide (see discussion 20 of "discontinuous epitope", below); accordingly, the present invention contemplates that the polypeptide of the vaccine may include at least one amino acid sequence of a zona pellucida protein that displays a functionally homologous epitope.

25 An amino acid sequence displaying an epitope for an available contraceptive antibody may be selected from all the sequences in a zona pellucida protein using a known contraceptive antibody. For example, a contraceptive antibody may be used to isolate a peptide displaying 30 its epitope from a proteolytic digest of a zona pellucida protein by means of affinity chromatography methods that are well known in the art.

Alternatively, a DNA sequence encoding an amino acid sequence which displays an epitope for a contraceptive antibody may be isolated by standard genetic engineering approaches. These involve screening of clones of 35 fragments of a gene for a zona pellucida protein for the ability to express an amino acid sequence that binds the contraceptive antibody. In addition, if sufficient zona

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proteins can be produced by standard recombinant DNA techniques, it may be possible to determine the 3-dimensional structure by standard biochemical techniques (e.g. nuclear magnetic resonance, and X-ray diffraction).

5 Yet another way to identify an amino acid sequence that displays the epitope of a contraceptive antibody is to employ the well known strategy of chemical synthesis of every distinct peptide that could possibly display an antibody epitope. For instance, technology is
10 commercially available for the rapid synthesis and antibody reactivity testing of all peptides of six amino acids that occur sequentially in the sequence of a protein and overlap by one amino acid. In the practice of the present invention, the sequences to be synthesized are determined
15 advantageously from the nucleotide sequence of a cloned gene for a zona pellucida protein.

In another embodiment of this aspect of the present invention, the amino acid sequence that displays the epitope for a contraceptive antibody in the vaccine
20 may be some type of analog of the amino acid sequence for that epitope on the zona pellucida protein.

One type of analog that this embodiment includes is a synthetic peptide known as a "mimotope" by H. M. Geysen, the inventor of the technology used to create such
25 analogs, for which kits of materials are now commercially available. In a substantial number of cases, this synthetic epitope generation approach produces amino acid sequences that are functional analogs of known epitopes for a given antibody, and these analogs can induce other
30 antibodies that recognize the same epitope as the original selected antibody. These analog sequences, however, usually do not contain the amino acids in the natural amino acid sequence that displays the selected epitope. Thus, this type of analog sequence mimics a naturally
35 occurring structure that displays an epitope, hence, the term "mimotope". An important feature of this particular aspect of this embodiment of the present invention is that it is not necessary to identify the natural amino acid sequence displaying the epitope of the desired contracep-

tive antibody; in fact, this method can produce small peptide analogs of natural epitopes comprising amino acids located in distinct positions of a protein that are separated by many amino acids (i.e., so-called "discontinuous epitopes" as opposed to those epitopes encoded by a single short continuous amino acid sequence).

In the term "analog", this aspect of the present invention also contemplates the application of well known principles of sequence conservation during the evolution of protein families to identify epitopes for contraceptive antibodies in a selected zona pellucida protein for which such antibodies are not yet available. If the amino acid sequence of this zona pellucida protein is highly homologous to that of related protein from another species, and if epitopes for such contraceptive antibodies have been defined in the sequence of this latter protein, then the general structural homology between the two proteins may be used to indicate those sequences in the selected protein that display epitopes for contraceptive antibodies that are analogous to those known for the second protein.

In other words, when two short, distinct amino acid sequences are known to occupy the same position in two proteins of substantially homologous structure (i.e., overall amino acid sequence and, consequently, three-dimensional conformation), then if one of the two sequences displays an epitope for an antibody with a particular biological effect, the other sequence has a high probability of displaying epitopes for other antibodies with the same biological effect. According to this aspect of this invention, a known epitope for a contraceptive antibody is embodied by an amino acid sequence identified in a mouse ZP3 protein by screening cloned fragments of a cloned DNA for expression of suitable epitopes, and one analog of this amino acid sequence is embodied by the sequence of amino acids that occupies the homologous position in the human ZP3 protein. A second epitope for a known contraceptive antibody is embodied by an amino acid sequence identified in a mouse ZP2 protein as above and one analog of this amino acid sequence is embodied by the sequence of

amino acids which occupies the homologous position in the human ZP3 protein. This human analog of a mouse ZP3 or ZP2 epitope (which also may be considered to be a "homologue" of that epitope), is to be incorporated into a 5 vaccine for use in human beings, of course, according to the alloimmunization aspect of the present invention.

It is understood that chemically synthesized peptides may be used advantageously as polypeptides of the present invention, especially since the synthesis of such 10 peptides comprising 30 to 50 or even more amino acids can now be achieved on scales sufficient for vaccine purposes (in batches of 1 gram or more, for example). One such synthetic peptide is embodied by a mouse ZP3 peptide and a mouse ZP2 peptide that are described below.

15 It should be particularly noted that the polypeptides of the present invention do not include idiotypic antibodies or large fragments of such antibodies, since the disadvantages of using such polypeptides to present epitopes of zona pellucida proteins has been 20 discussed above in the Background in regard to prior art on such antibodies. However, the present invention does contemplate smaller polypeptides comprising mainly those amino acid sequences of such idiotypic antibodies that actually comprise the analog of the original zona pelluci- 25 da protein epitope.

Further, as will be appreciated from the Background discussion of immunogenicity of polypeptides, the immunogenicity of polypeptides or peptides of the present invention in terms of raising higher titers of contraceptive 30 antibodies with greater affinities for their epitopes, particularly such immunogenicity of small (synthetic) peptides, may be enhanced advantageously by covalent coupling to another polypeptide or peptide, especially to another amino acid sequence displaying a T- 35 cell epitope.

In addition, it will be appreciated that, as is customary for vaccines, the polypeptides of the present invention will be delivered in a pharmacologically acceptable vehicle. Vaccines of the present invention may also

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advantageously comprise effective amounts of immunological adjuvants that are known to enhance the immune response to immunogens in general, particularly adjuvants that enhance the immunogenicity of small synthetic peptides.

5 In another aspect, the present invention further relates to certain DNA segments that encode mouse ZP3 or ZP2 proteins and human ZP3 or human ZP2 proteins. This invention also relates to cultures of recombinant cells containing a DNA segment of this invention and to methods
10 for the synthesis and isolation of polypeptides and peptides of this invention.

The present invention also relates to recombinant DNA molecules comprising a DNA segment of this invention and a vector. A number of vectors may be
15 utilized such as, for example, the vaccinia virus.

In particular, the present invention includes a contraceptive vaccine for use in a mammalian female comprising a polypeptide which consists essentially of the mouse zona pellucida 3 (ZP3) amino acid sequence Cys-Ser-
20 Asn-Ser-Ser-Ser-Ser-Gln-Phe-Gln-Ile-His-Gly-Pro-Arg-Gln or a homologous mammalian amino acid sequence derived from a homologous region of a ZP3 protein. The mammalian amino acid sequence is included in a zona pellucida protein originating from the species in which the vaccine is used.
25 The vaccine also includes a pharmacologically acceptable vehicle. It must be noted that portions of the sequence may also be utilized in the vaccine.

The homologous mammalian amino acid sequence in the vaccine may be, for example, Cys-Gly-Thr-Pro-Ser-His-
30 Ser-Arg-Arg-Gln-Pro-His-Val-Met-Ser-Gln. This sequence is derived from a human ZP3 protein. It should be noted that portions of the 16 amino acid sequence may be utilized in the vaccine.

Additionally, the present invention relates to
35 a contraceptive vaccine for use in a mammalian female comprising a polypeptide which consists essentially of the mouse zona pellucida 2 (ZP2) amino acid sequence Ile-Arg-Val-Gly-Asp-Thr-Thr-Asp-Val-Arg-Tyr-Lys-Asp-Asp-Met or a homologous mammalian amino acid sequence derived from a

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homologous region of a ZP2 protein. The mammalian amino acid sequence is included in a zona pellucida protein originating from the species in which the vaccine is used. The vaccine also includes a pharmacologically acceptable 5 vehicle. Portions of the 16 amino acid sequence may be utilized in the vaccine.

The homologous mammalian amino acid sequence referred to above may be, for example, Ile-Arg-Val-Met-Asn-Asn-Ser-Ala-Ala-Leu-Arg-His-Gly-Ala-Val-Met and is 10 derived from a human ZP2 protein. It should be noted that portions of the 16 amino acid sequence may also be utilized in the vaccine.

Each of the above-vaccines may include an effective amount of an adjuvant. Furthermore, the mammalian female may be a cat, a dog, a pig, a cow, or a woman. It is important to note that the polypeptide is derived 15 from the same species to which it is administered in vaccine form.

The present invention also relates to a contraceptive vaccine comprising a polypeptide which consists 20 essentially of a synthetic peptide corresponding to the mouse zona pellucida (ZP3) amino acid sequence Cys-Ser-Asn-Ser-Ser-Ser-Gln-Phe-Gln-Ile-His-Gly-Pro-Arg-Gln or a synthetic peptide corresponding to a homologous mammalian 25 amino acid sequence derived from a homologous region of a ZP3 protein, for binding of an antibody that inhibits fertilization of an egg by a sperm. The mammalian amino acid sequence, as noted above, is included in a zona pellucida protein originating from the species in which 30 said vaccine is used. The vaccine may further comprise a pharmacologically acceptable vehicle. Portions of the 16 amino acid sequence may also be used.

The homologous mammalian amino acid sequence may be, for example, Cys-Gly-Thr-Pro-Ser-His-Ser-Arg-Arg-Gln-35 Pro-His-Val-Met-Ser-Gln and is derived from a human ZP3 protein. Portions of the homologous sequence may be utilized in the vaccine.

The present invention also relates to a contraceptive vaccine comprising a polypeptide which consists

essentially of a synthetic peptide corresponding to the mouse zona pellucida (ZP2) amino acid sequence Ile-Arg-Val-Gly-Asp-Thr-Thr-Asp-Val-Arg-Tyr-Lys-Asp-Asp-Met or a synthetic peptide corresponding to a homologous mammalian amino acid sequence derived from a homologous region of a ZP2 protein, for binding of an antibody that inhibits fertilization of an egg by a sperm. The mammalian amino acid sequence is included in a zona pellucida protein originating from the species in which the vaccine is used.

10 The vaccine may further comprise a pharmacologically acceptable vehicle. It should be noted that portions of the sequence shown above may be utilized in the vaccine.

The homologous mammalian amino acid sequence may be, for example, Ile-Arg-Val-Met-Asn-Asn-Ser-Ala-Ala-Leu-15 Arg-His-Gly-Ala-Val-Met which is derived from a human ZP2 protein. Portions of this sequence may be utilized in the vaccine.

Additionally, the present invention also includes a DNA segment encoding the mouse ZP3 protein or a portion thereof, a DNA segment encoding the mouse ZP2 protein or a portion thereof, a DNA segment encoding the human ZP3 protein or a portion thereof, and a DNA segment encoding the human ZP2 protein or a portion thereof.

The invention also encompasses a recombinant DNA molecule comprising a DNA segment encoding the human ZP3 or human ZP2 protein, or a portion of each protein, and a vector. Additionally, the invention includes cultures of host cells transformed or transfected with the recombinant DNA molecules or constructs.

30 The invention also includes a method of producing at least a portion of a human ZP3 or human ZP2 protein comprising culturing the above-cells under conditions such that the protein is produced and isolating said protein from culture media or from the cells.

35 The invention also includes an antibody specific for a protein having the amino acid sequence of the human ZP3 or ZP2 protein or a portion thereof. This antibody inhibits fertilization of a human oocyte by a sperm.

Furthermore, the invention also includes the

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purified proteins encoded by the DNA segments referred to above. All U.S. patents and publications referred to herein are hereby incorporated by reference.

5 The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples and Figures included therein.

Brief Description of the Figures

10

FIGURE 1: Comparison of the Secondary Structures of Mouse and Human ZP2 Proteins and of Mouse and Human ZP3 Proteins.

15 The hydropathicity of the 713 amino acid mouse ZP2 (Liang et al., Mol. Cell. Biol. 10: 1507-15 (1990) and 745 amino acid human ZP2 (Liang et al., Dev. Biol. 156: 399-408 (1993)), determined by the Kyte and Doolittle algorithm (Kyte et al., J. Mol. Biol. 157: 105-32 (1982)), indicates the overall similarity of the two proteins.

20 Both have major hydropathic peaks in their signal peptides and near their carboxyl termini. The hydropathicity of the 424 amino acid mouse ZP3 (Ringuette et al., Dev. Biol. 127: 287-295 (1988)) and 424 amino acid human ZP3 (Chamberlin et al., 87: 6014-18 (1990)), determined by the 25 Kyte and Doolittle algorithm (Kyte et al., supra), indicates the overall similarity of these two proteins. Both have major hydropathic peaks in their signal peptides and near their carboxyl termini.

30 **FIGURE 2:** The Definition of a Potential Zona Pellucida Peptide for Use as a Contraceptive Vaccine by Screening a ZP3 Epitope Library with a Monoclonal Antibody Specific to ZP3.

35 (A) Schematic representation of the 1317 nucleotide ZP3 mRNA. The single 1272-nt open reading frame is indicated by an open bar. The lines below the mRNA represent eight positive cDNA clones isolated from the ZP3 epitope library by the monoclonal antibody to ZP3. The clones are aligned on the ZP3 cDNA and the hatched bar

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indicates the sequence common to all positive clones.

(B) The DNA sequence of the overlapping regions among the eight positive clones and the corresponding amino acid sequence (**bold**) are shown. The one additional 5 COOH-terminal and eight additional NH₂-terminal amino acids shown flanking the epitope were included in the peptide used for immunization.

(C) Hydrophilicity of the deduced 424-amino acid ZP3 protein was plotted with a seven-residue moving 10 average. Horizontal filled-in bars beneath the hydrophilicity indicate amphipathic α helical segments predicted by an 11-residue moving average. The speckled vertical bar represents the 16-amino acid peptide shown in 7B) that was used to immunize experimental animals (Millar et al., 15 Science 246: 935-38 (1989)).

FIGURE 3: Localization of Two Monoclonal Antibody Binding Sites on Mouse ZP2 and ZP3.

(A) Hydrophilicity of the 713-amino acid ZP2 20 protein plotted with a seven-residue moving average. Horizontal filled-in bars beneath the hydrophilicity plot indicate amphipathic α helical segments predicted by an 11-residue moving average. The speckled vertical bar represents the 16-amino acid peptide that is the binding 25 site of a monoclonal antibody specific to mouse ZP2.

(B) Hydrophilicity of the 424-amino acid ZP3 protein plotted with a seven-residue moving average. Horizontal filled-in bars beneath the hydrophilicity plot indicate amphipathic α helical segments predicted by an 30 11-residue moving average. The speckled vertical bar represents the 7-amino acid peptide that is the binding site of a monoclonal antibody specific to mouse ZP3.

FIGURE 4: Alignment of the Mouse ZP3 and ZP2 Epitopes 35 with the Homologous Portions of the Human ZP2 and ZP3 Proteins.

(A) Mouse ZP3 amino acids 328-343 aligned with human ZP3 amino acids 327-342.

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(B) Mouse ZP2 amino acids 114-129 aligned with human ZP2 amino acids 118-133.

Detailed Description of the Invention

5 The present invention relates in part to DNA segments having sequences that encode mouse and human ZP3 and ZP2 proteins. An embodiment of this aspect of the invention includes cDNA and genomic clones that encode at least a portion of the complete nucleotide sequence of the
10 mouse ZP3 mRNA and the protein encoded thereby which has been described by the present inventor in Example 1, below, and has been published (Ringuette et al., Dev. Biol. 127:287-95 (1988); Chamberlin et al., Dev. Biol. 131:207-14 (1989)).

15 A second embodiment of this aspect of the invention includes cDNA and genomic clones that encode at least a portion of the complete nucleotide sequence of the mouse ZP2 mRNA and the protein encoded thereby which has been described by the present inventor in Example 1, below, and has been published (Liang et al., Mol. Cell. Biol. 10:1507-15 (1990)).

20 A third embodiment of this aspect of the invention includes cDNA and genomic clones that encode at least a portion of the complete nucleotide sequence of the human ZP3 mRNA and the protein encoded thereby which has been described by the present inventor in Example 1, below, and has been published (Chamberlin et al., Proc. Natl. Acad. Sci. USA 87:6014-18 (1990)).

25 A fourth embodiment of this aspect of the invention include cDNA and genomic clones that encode at least a portion of the complete nucleotide sequence of the human ZP2 mRNA and the protein encoded thereby which has been described by the present inventor in Example 1, below, and has been published (Liang et al., Dev. Biol. 156:399-408 (1993)).

30 A summary of this information follows:

Genomic Organization and Conservation of the Zona Pellucida Genes: Mouse *Zp-2* and *Zp-3* are each present in a single copy in the mouse genome and are present on differ-

ent chromosomes. *Zp-2* is located on chromosome 7, 11.3 ± 3.2 cM distal to the *Tyr* locus and *Zp-3* is located on chromosome 5, 9.2 ± 2.9 cM distal to the *Gus* locus (Lunsford et al., Genomics 6:184-87 (1990)). Mouse *Zp-2* 5 contains 18 exons that range in size from 45bp to 190bp separated by 17 introns (81bp to 1490bp) and spans 12.1-kbp of DNA (Liang et al., supra (1990)). The 8.6-kbp long mouse *Zp-3* gene contains 8 exons ranging in size from 92bp to 338bp and has introns whose lengths are between 125bp 10 and 2320bp (Chamberlin et al., Dev. Biol. 131:207-14 (1989)). The intron-exon boundaries of both genes contain consensus splice donor/acceptor sites (Breathnach et al., Annu. Rev. Biochem. 50:349-83 (1981)).

The genes encoding *ZP2* and *ZP3* are conserved 15 among mammals. Taking cross-hybridization of nucleic acid sequences as a criteria, the degree of conservation of *Zp-3* is variable with pig and rabbit being less related to mouse than rat, dog, cow and human zona genes (Ringuette et al., Proc. Natl. Acad. Sci. U.S.A. 83:4341-45 (1986)). 20 The human homolog of *Zp-2* and *Zp-3* have been isolated using standard genetic engineering approaches well known in the art by virtue of their homology to the previously isolated murine *ZP2* and *ZP3* cDNAs. The human *ZP2* gene is composed of 19 exons whose nucleic acid sequence is 70% 25 the same and encodes a 745 amino acid protein that is 60% identical to that of its mouse counterpart (Liang et al., Dev. Biol. 156:399-408 (1993)). The mouse and human *ZP3* genes each contain 8 exons. The coding sequence of the mouse and human genes are 74% the same and each encodes a 30 424 amino acid peptide that is 67% identical (Chamberlin et al., Proc. Natl. Acad. Sci. U.S.A. 87:6014-18 (1990)).

ZP2 mRNA and Protein: The structure of mouse *ZP2* was deduced from near-full-length cDNA clones and genomic 35 clones containing exon 1. The *ZP2* mRNA is 2201 nt long with very short 5' (30 nt) and 3' (32 nt) untranslated regions. A transcript of approximately 2.4-kbp is observed by Northern blot analysis of oocyte RNA suggesting that *ZP2* mRNA contains a poly(A) tail of approximately 200

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nt (Liang et al., Mol. Cell. Biol. 10:1507-15 (1990)). ZP2 mRNA has a single open reading frame of 2139 nt initiated at an ATG within the ANNATG motif associated with vertebrate initiator codons (Kozak, Cell 44:283-93 5 (1986); Cavener, Nucleic Acids Res. 15:1353-61 (1986)). The open reading frame encodes a polypeptide of 713 amino acids with a molecular weight of 80,217 daltons, the amino acid composition of which is 10.8% acidic, 9.5% basic, 10.2% aromatic and 34.8% hydrophobic.

10 The first 34 amino acids of the deduced polypeptide are absent from the N-terminal amino acid sequence obtained from SDS-PAGE purified ZP2 protein and presumably represent a signal peptide. The amino acids at the -1 and -3 position from the presumptive signal peptidase cleavage 15 site are Ser and Asn, respectively. The positions of these two amino acids are similar to other eukaryotic signal peptidase cleavage sites (Perlman et al., J. Mol. Biol. 167:391-409 (1983)) and are in accordance with the (-3, -1) rule of signal peptidase cleavage sites proposed 20 by von Heijne (Von Heijne, J. Mol. Biol. 184:99-105 (1985); Von Heijne, Nucleic Acids Res. 14:4683-90 (1986)). The resultant core polypeptide secreted into the extracellular matrix would have a molecular weight of 76,373 daltons. The ZP2 amino acid sequence contains seven 25 possible N-linked glycosylation sites (Asn-X-Ser/Thr), and more than 100 potential O-linked glycosylation sites (Liang et al., supra (1990)).

The human and mouse ZP2 mRNAs and proteins are well conserved. The human ZP2 mRNA contains an open 30 reading frame of 2235 nt that can code for a polypeptide of 82,356 daltons containing 745 amino acids (10.2% acidic, 11.5% basic, 9.4% aromatic and 50.3% hydrophobic). Human and mouse ZP2 amino acid sequences are 60.7% identical. Examination of human ZP2 protein revealed a potential 35 signal peptidase cleavage site which contains amino acids at the -1 and -3 positions that are in accordance with the (-3, -1) rule proposed by von Heijne (Von Heijne, J. Mol. Biol. 184:99-105 (1985); Von Heijne, Nucleic Acids Res. 14:4683-90 (1986)). Cleavage at the

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presumptive signal peptidase site would give rise to a signal sequence of 38 amino acids (4 residues longer than mouse ZP2) and a resultant protein with a predicted molecular mass of 78,200 daltons. The deduced polypeptide 5 chain contains six potential N-linked glycosylation sites (Asn-X-Ser/Thr), four of which are conserved in the mouse ZP2 polypeptide. The predicted hydropathicity of the human and mouse ZP2 proteins are quite similar, reflecting both amino acid identity and conservative amino acid 10 substitutions (Figure 1). The conservation of all 20 cysteine residues in the mature human and mouse proteins suggests that at least some of these residues participate in disulfide bonds important for tertiary structure. An additional exon found in human ZP2 encodes a 28 amino acid 15 hydrophilic region (residues 671-698) near the carboxyl terminus.

ZP3 mRNA and Protein: Primer extension studies and S1 nuclelease protection assays were used to define the 5' 20 terminus of the mouse ZP3 mRNA. Similar to ZP2 mRNA, the 1317 nt ZP3 mRNA has short 5' (29 nt) and 3' (16 nt) untranslated regions. The latter is so abbreviated that the TAA termination codon is embedded within the consensus AATAAA polyadenylation signal (Ringuette et al., Dev. 25 Biol. 127:287-95 (1988)). It is not clear what the role, if any, that these short untranslated regions play in gene expression nor whether they are important for processing ZP2 and ZP3 transcripts. This short untranslated region is a characteristic of both ZP2 (mouse and human) and ZP3 30 (mouse and human) mRNAs. The mouse ZP3 mRNA in oocytes is 1.5-kb, indicating that it has a poly(A) tail of 200 nt, and is indistinguishable in size from that of rat and rabbit (Ringuette et al., supra (1988)). Taken together, these data suggest that the overall structure of ZP3 mRNA 35 is conserved among mammals. The polypeptide deduced from the single open reading frame of mouse ZP3 mRNA is 46,307 daltons consisting of 424 amino acids (9% acidic, 7.3% basic, 7.5% aromatic and 31.4% hydrophobic). The N-terminal amino acid of the secreted glycoprotein is

blocked to Edman degradation, but using the sliding window/matrix scoring method of von Heijne (Von Heijne, supra (1985); Von Heijne, supra (1986)), a potential signal peptide of 22 amino acid has been identified 5 (Ringuette et al., supra (1988)). The resultant secreted protein would have a molecular weight of 43,943 daltons, consistent with the reported 44,000 dalton ZP3 core protein (Bleil et al., Dev. Biol. 76:185-202 (1983)).

The human and mouse ZP3 mRNAs and proteins are 10 well conserved. The human ZP3 mRNA contains an open reading frame of 1272 nt that can code for a polypeptide of 47,032 daltons containing 424 amino acids (12% acidic, 8% basic, 7% aromatic and 32% hydrophobic). Human and mouse ZP3 amino acid sequences are 67% identical. Examination 15 of human ZP3 protein revealed a potential signal peptidase cleavage site which contains amino acids at the -1 and -3 positions that are in accordance with the (-3, -1) rule proposed by von Heijne (Von Heijne, supra (1985); Von Heijne, supra (1986)). Cleavage at the 20 presumptive signal peptidase site would give rise to a signal sequence of 22 amino acids and a resultant protein with a predicted molecular mass of 44,399 daltons. The deduced polypeptide chain contains four potential N-linked glycosylation sites (Asn-X-Ser/Thr), three of which are 25 conserved in the mouse ZP3 polypeptide. The predicted hydropathicity of the human and mouse ZP3 proteins are quite similar, reflecting both amino acid identity and conservative amino acid substitutions (Figure 1). The conservation of all 13 cysteine residues in the mature 30 human and mouse proteins suggests that at least some of these residues participate in disulfide bonds important for tertiary structure.

Conservation of Zona Protein Structure: The data in 35 Figure 1 clearly show the high homology of the mouse and human ZP3 and ZP2 sequences, as would be expected from the extensive nucleic acid hybridization observed between mouse ZP3 cDNA and genomic DNAs from a variety of other mammalian species (see Example 2). From this structural

homology data, and further standard analyses thereof (e.g., predictions of secondary structure, hydropathicity, or surface accessibility), it would be apparent to one of average skill in the art of protein structure and immunology that the mouse and human ZP3 proteins must also exhibit throughout their entire sequences, an extremely high level of functional homology with respect to locations that are able to induce and bind contraceptive antibodies. Thus, although epitopes for contraceptive antibodies on each protein may comprise short amino acid sequences which are not precisely conserved between the two proteins, the human sequences corresponding to such epitopes on the mouse protein are also expected to induce functionally homologous antibodies, even though the mouse and human antibodies might only recognize their respective alloantigens.

It will be obvious, of course, to one of ordinary skill in the art of genetic engineering, that the above ZP3 and ZP2 sequences may vary slightly (i.e., be mutated) from one inbred mouse strain to another, or from one individual in an outbred population (e.g., one human being) to another, without materially affecting the immunological character of the corresponding zona pellucida protein and, therefore, without departing from the scope of the DNAs of the present invention as conveyed, for example, by the use of the terms "the mouse ZP3 protein" or "the human ZP3 protein" or "the mouse ZP2 protein" or "the human ZP2 protein".

The DNA segments of the present invention variously enable development of different embodiments of the main aspect of the present invention, namely contraceptive vaccines for use in a mammalian female comprising a polypeptide which includes an amino acid sequence that is selected to display at least one epitope for binding of an antibody that inhibits fertilization of an oocyte by a sperm. This contraceptive antibody epitope is an epitope for which there is a functional homolog displayed on a zona pellucida protein that originates from the species in which the vaccine is used. The zona pellucida protein

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displaying the functionally homologous epitope advantageously is either a ZP3 protein or a ZP2 protein or a ZP1 protein.

A principal embodiment of this aspect of this invention are two contraceptive antibody epitopes that are displayed either on the mouse ZP3 or the mouse ZP2 protein. Synthetic peptides containing either of these epitopes, when coupled to a carrier protein, for example, KLH, will elicit antibodies after alloimmunizations that react with the zona pellucida. FIGURE 2 outlines the definition of the mouse ZP3 epitope for a contraceptive antibody, which is described in further detail in Example 3, below. A similar strategy was employed to define the mouse ZP2 epitope for a second contraceptive antibody. The ZP2 and ZP3 epitopes along with their human homologues are shown in FIGURE 4.

In brief, a cDNA encoding ZP3 was randomly fragmented and 200-500 bp fragments were cloned into the expression vector λ gt11. This epitope library was screened with the aforementioned anti-ZP3 contraceptive monoclonal antibody and the positive clones were used to map a seven amino acid epitope (amino acids 336-342) on mouse ZP3 recognized by the antibody. The homologous region on human ZP3 is contained in amino acids 335-341.

In a similar fashion, a cDNA encoding ZP2 was randomly fragmented to create a second epitope library which was screened with the aforementioned anti-ZP2 contraceptive monoclonal antibody. Positive clones were used to define a 16 amino acid epitope (amino acids 114-129) on mouse ZP2 recognized by the antibody. The homologous region on human ZP2 is contained in amino acids 118-133.

Of course, it must be noted that a shorter portion of the 7 amino acid sequence that displays the ZP3 epitope or the 16 amino acid sequence that displays the ZP2 epitope might also be an effective peptide for purposes of the present invention. Furthermore, certain analogues (e.g., those sequences with ends that are chemically modified to neutralize charges) might provide effective

peptides for the practice of the present invention.

Female mice were immunized with a synthetic peptide containing the ZP3 epitope, as described in Example 4, and the resultant circulating anti-ZP3 antibodies bound to the oocytes of immunized animals producing long-lasting contraception. As evidence that the effectiveness of alloimmunization with a zona pellucida peptide is not limited to the ZP3 protein, additional female mice were immunized with a synthetic peptide containing the ZP2 epitope, as described in Example 4. This vaccination also elicited antibodies that bound to the zona pellucida proteins.

The reversibility of the contraceptive effect, described in Example 4, can be accounted for by resting oocytes entering into the growth phase and synthesizing a zona pellucida in the presence of low-levels of circulating anti-zona antibodies which appear to decline after immunization with the vaccine is terminated. When ovulated, these oocytes would be coated lightly, if at all, with anti-zona antibodies and would, therefore, be capable of being fertilized.

Studies have demonstrated that repeated immunization of female mice with a mouse ZP3 peptide-KLH conjugate results in long-term infertility in the majority of cases. The production of anti-zona pellucida antibodies occurs despite the fact that the zona peptide is a self antigen (alloantigen). Immune tolerance has been postulated to occur in the neonatal period of development and involves both the functional inactivation of B cells and the deletion of T cells which recognize self antigens. The lack of detectable zona proteins in the ovary until 2-3 days after birth, or their inaccessibility to the developing immune system, may account for the continued presence of lymphocytes capable of recognizing at least one ZP3 epitope.

In regard to the eventual reversibility of the contraceptive immunization, it is curious that having mounted an immunological response against the ZP3 peptide-KLH conjugate, the immune system does not continue to be

stimulated by the endogenous ZP3 protein. The following hypotheses may account for this phenomenon in whole or in part, and, therefore, aid in understanding the present invention; but these theoretical explanations should not 5 be construed to limit the scope of the present invention in any way. Nevertheless, it may be speculated that one or more of the following may be involved in the reversibility of the contraceptive immunization: 1) The localization of the zona proteins uniquely to the ovary coupled 10 with the lack of capillaries beyond the basement membrane surrounding the follicles, may physically preclude lymphocytes from interacting with and being stimulated by the zona pellucida; 2) The 16 amino acid ZP3 peptide portion of the immunogen provides a B-cell epitope but may not 15 contain T-cell epitopes (which may, instead, be provided by the KLH moiety) to stimulate helper T-cell functions. Thus, the endogenous ZP3 protein, although containing the same ZP3 peptide, would not contain the T-cell epitopes of the carrier protein that, according to this hypothesis, 20 could be important for mounting an anti-ZP3 peptide response; 3) The ovary may be part of an immunologically protected region and mechanisms that suppress the immunological rejection of the embryo (which contains paternal and, thus, foreign antigens) also function in the ovary.

25 It is particularly important to note that immunization with the ZP3 peptide vaccine did not result in either structural or functional abnormalities of the mouse ovary (viz normal histology and the ability of vaccinated females to subsequently have litters). In this 30 regard, of course, the use of a synthetic ZP3 peptide as a vaccine precludes any possible minor contamination with other ovarian immunogens. In addition, the physical barrier of the follicular basement membrane and the extra-cellular site of the zona protein may contribute to the 35 absence of an immunocytotoxic response in the ovary. It should be noted that a nearby, partially overlapping T cell epitope is able to elicit an inflammatory response in some but not other inbred strains of mice known to be susceptible to autoimmune oophoritis (Rhim et al., J.

5 Clin. Invest. 89: 28-35 (1992)). The potential to elicit an antibody response in the absence of an ovarian inflammatory response may be an additional advantage of this invention (Millar et al., Targeting of zona pellucida for
5 immunocontraception, in Immunology of Reproduction, Naz, R.K, (ed) pp. 293-313 (1993)).

10 The mouse ZP3 epitope recognized by the monoclonal antibody used to develop this vaccine is not detected immunologically in hamster, guinea pig, cat or
10 dog ovaries. Thus, this ZP3 peptide would not be expected to act as a contraceptive in other mammalian species, including human beings, although the ability of this antibody to bind to the human ZP3 protein has not been tested. However, the strategy of the present invention of
15 using vaccination with "self" zona peptides can be applied to other species by taking advantage of the highly conserved nature of the zona genes among mammals. As noted above, the human homologues of the mouse ZP3 and ZP2 genes have been characterized, and the high degree of structural
20 homology is one indication of comparable functional homology in relation to epitopes for contraceptive antibodies.

25 Accordingly, using the deduced primary amino acid sequence of the human ZP3 and ZP2 proteins, by the practice of the present invention without undue experimentation, it is believed that one of ordinary skill in the art of polypeptide structure and immunology can identify in the human or other mammalian ZP3 and ZP2 proteins the region homologous to the mouse ZP3 and ZP2 peptides
30 described herein. Alternatively, one of such skill may use computer algorithms to predict additional epitopes which may be potential immunogens (T.P. Hopp and K.R. Woods, Proc. Natl. Acad. Sci. USA 78:3824 (1981); H. Maragalit, et al. J. Immunol. 138:2213 (1987); J.B.
35 Rothbard and W.R. Taylor, EMBO J. 7:93 (1988)), or test a large array of peptides representative of the polypeptide chain for epitopes of contraceptive antibodies using well known methods (H.M. Geysen et al. Proc. Natl. Acad. Sci. USA 81:3998 (1984); R.A. Houghten, Proc. Natl. Acad. Sci.

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USA 82:5131 (1985); H.M. Geysen, et al. Science 235:1184 (1987); E. Norrby, et al. Proc. Natl. Acad. Sci. USA 84:6572 (1987)).

Further, as noted previously, one skilled in the
5 art of synthetic peptide vaccines can also develop
"mimotopes" of epitopes to available contraceptive anti-
bodies. According to this approach, first, the ability of
any desired antibody to bind to essentially every possible
10 sequence of two amino acids that naturally appear in
proteins is tested. Upon identification of a pair of
amino acids with detectable binding of the antibody, the
sequence surrounding those two amino acids is progressive-
ly and systematically varied, by the inclusion of each of
15 the naturally occurring amino acids as well as some amino
acids not found in natural proteins, until continued
testing of antibody binding identifies a short peptide
displaying an epitope with sufficient affinity for the
selected antibody to be used for the desired purpose.

Thus, the approach of this invention of
20 alloimmunization with epitopes of zona proteins is expect-
ed to have wide application in the design of future
contraceptive vaccines for the control of mammalian
populations.

The present invention can be illustrated by the
25 use of the following non-limiting examples.

Example 1: Determination of the Primary Structure of
Mouse and Human Zona Pellucida Proteins by Cloning and
Characterizing the Mouse and Human ZP3 and ZP2 Genes.

30 A cDNA library was made from poly(A)⁺ RNA
isolated from mouse ovaries tissues using techniques
standard to the field (Ringuette et al., Proc. Natl. Acad.
Sci. USA 83:4341-45 (1986)). Eco RI linkers were added to
the ends of the cDNAs and the library was cloned into Eco
35 RI site of lambda gt11. The library was packaged and used
to infect E. coli Y1090 cells which were mixed with
agarose and plated in agar-filled petri dishes using
standard techniques. The lytic phase was induced by a
temperature shift from 37°C to 42°C. Nitrocellulose

filters, impregnated with isopropyl β -D-thiogalactoside, were used to induce expression of β -galactosidase fusion proteins by λ gt11 recombinants. Those containing ZP2 or ZP3 epitopes were detected with a rabbit antisera that had 5 been raised against heat solubilized mouse zonae pellucidae. The positive clones were plaque purified and tested for their ability to express fusion proteins that reacted with rat monoclonal antibodies specific to either ZP2 or ZP3. Two λ gt11 recombinants reacted with a monoclonal 10 antibody specific to ZP3 (Ringuette et al., supra (1986); Ringuette et al., Dev. Biol. 127:287-95 (1988)) and one λ gt11 recombinant reacted with a monoclonal antibody specific to ZP2 (Liang et al., Mol. Cell. Biol. 10:1507-15 (1990)).

15 Mouse ZP3: The cDNA insert from a single λ gt11 clone was subcloned (pZP3.1) and used to rescreen the library to obtain additional cDNAs. The 5' most 46 nt were determined from a genomic clone and the transcription initiation site was determined by procedures standard to 20 the field (Ringuette et al., supra (1988)). These sequences were used to determine the structure of the mouse ZP3 mRNA and the resultant protein. The ZP3 mRNA is a 1317 nt polyadenylated transcript that contains a single open reading frame encoding a 424 amino acid polypeptide 25 chain with a predicted mass of 46,307 Da. The identity of the cDNA clone was confirmed by comparison of its deduced amino acid sequence with that of a 20 amino acid sequence obtained from an internal peptide of purified ZP3 protein. A predicted signal peptidase cut site after amino acid 17 30 would result in a polypeptide with a mass of 43,943 Da (Ringuette et al., supra 1988). The 83,000 Da mass of the native, secreted ZP3 sulfated glycoprotein reflects post-translational modifications of the polypeptide chain. Additional characteristics of this protein have been noted 35 above.

Mouse ZP2: The cDNA insert from a single λ gt11 clone was subcloned (pZP2.1) and used to rescreen the library to obtain additional cDNAs that contained sequences that encoded the entire polypeptide chain (Liang et

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al., Mol. Cell. Biol. 10:1507-15 (1990)). The 5' most 21 nt were determined from a genomic clone, and the transcription initiation site was determined by procedures standard to the field. These sequences were used to 5 determine the structure of the mouse ZP2 mRNA and the resultant protein. The ZP3 mRNA is a 2201 nt polyadenylated transcript that contains a single open reading frame encoding a 713 amino acid polypeptide chain with a predicted mass of 80,217 Da. The identity of the 10 clone was confirmed by comparison of its deduced amino acid sequence with that of a 16 amino acid sequence from a N-terminal peptide and with that of a 10 amino acid sequence obtained from an internal peptide of purified ZP2 protein. The first 34 amino acids represent a signal 15 peptide, the cleavage of which would result in a polypeptide with a mass of 76,373 Da (Liang et al., supra (1990)). The 120-140,000 Da mass of the native, secreted ZP2 sulfated glycoprotein reflects post-translational modifications of the polypeptide chain.

20

Example 2: Conservation of the Zona Pellucida Genes Among Mammals, Specifically Mouse and Human.

Mouse *Zp-3* genomic clones were isolated from a λ J1 library containing mouse B10A genomic DNA inserts by 25 screening with mouse ZP3 cDNA (Chamberlin et al., Dev. Biol. 131:207-14 (1989)). Characterization of two overlapping clones revealed that the single copy *Zp-3* gene contains 8 exons spanning 8.6-kbp. Exon sequences confirmed the previously described coding region of the ZP3 30 mRNA (Ringuette et al., Dev. Biol. 127:287-95 (1988); Chamberlin et al., supra (1989)). A mouse *Zp-2* genomic clone was isolated from the same λ J1 library by screening with mouse ZP2 cDNA (Liang et al., Mol. Cell. Biol. 10:1507-15 (1990)). The single copy *Zp-2* gene contains 18 35 exons spanning 12.1-kbp. Exon sequences confirmed the previously described coding region of the ZP2 mRNA (Liang et al., supra (1990)).

DNA was isolated from seven mammalian species: mouse, rat, rabbit, dog, pig, cow and human. After diges-

tion with a restriction enzyme (e.g., Bam H1) and transfer to a membrane by Southern blotting, the DNAs were probed with mouse ZP3 cDNA using standard techniques. Although stronger hybridization was detected with rat, dog, cow and 5 human than with rabbit and pig DNA, cross-hybridization was detected with DNA from all mammalian species (Ringuette et al., Proc. Natl. Acad. Sci. USA 83:4341-45 (1986)). Similar results were obtained using ZP2 cDNA probes. Mouse ZP3 cDNA probes cross-hybridized with rat 10 and rabbit ovarian poly(A)⁺ RNA on Northern blots and all three species have transcripts of similar size (Ringuette et al., supra (1988)). Taken together, these data suggest that the zona genes are well conserved among mammals. To further substantiate this hypothesis, human ZP2 and human 15 ZP3 genes and their RNA transcripts were isolated and characterized.

Human ZP3: A Charon 4A human genomic library was screened with mouse ZP3 cDNA under low stringency to allow cross-hybridization with the heterologous probe 20 (Chamberlin et al., Proc. Natl. Acad. Sci. USA 87:6014-18 (1990)). A single recombinant phage was isolated and characterized. This clone contained exons 1-5 of the human ZP3 gene. The remaining 6-8 exons were cloned from 25 genomic DNA using the polymerase chain reaction and oligonucleotide primers from human exons 6 and 8 (determined from human ZP3 cDNA, see below). The human ZP3 genes contains 8 exons, the sizes of which have near identity with those of mouse *Zp-3*, and the human gene spans approximately 18.3-kbp (Chamberlin et al., supra 30 (1990)).

Poly (A)⁺ RNA was isolated from a human ovary and used in a RT-PCR reaction (reverse transcription to make a single strand cDNA template, followed by exon specific oligonucleotide primers in the polymerase chain reaction) 35 to construct full-length cDNA clones representative of the human ZP3 transcript) (Chamberlin et al., supra (1990)). The human ZP3 transcript has a single 1272 nt open reading frame, the nucleic acid sequence of which is 74% identical to that of the mouse ZP3 transcript. The human transcript

5 encodes a 424 amino acid polypeptide ZP3 protein with a calculated molecular mass of 47,032 Da that is 67% identical to that of the mouse ZP3 protein. The hydropathicity profiles of the human and mouse ZP3 proteins are remarkably similar and reflect the conserved nature of the allowable amino acid substitutions (Chamberlin et al., supra (1990)). Additional characteristics of this protein have been noted above.

10 Human ZP2: A Charon 4A human genomic library was screened with mouse ZP2 cDNA under low stringency to allow cross-hybridization with the heterologous probe (Liang et al., Dev. Biol. 156: 399-408 (1993)). Three overlapping recombinant phages were isolated and characterized. These clones contained the entire 14.0-kbp human 15 ZP2 locus which is made up of 19 exons. Overall, these coding regions are 70% identical to those of mouse *Zp-2*. In addition, human ZP2 contains an extra exon of 84 bp (exon 18) that is not found in mouse ZP2 cDNA. Sequence analysis of mouse *Zp-2* intron 17 revealed a region of 76 20 bp that shares a 70% sequence homology with human ZP2 exon 18 (Liang et al., supra (1993)).

Poly (A)⁺ RNA was isolated from a human ovary and used in a RT-PCR reaction (reverse transcription to make a single strand cDNA template, followed by exon specific 25 oligonucleotide primers in the polymerase chain reaction) to construct cDNA clones representative of the human ZP2 transcript) (Liang et al., supra (1993)). In addition, human ovarian mRNA was used in the construction of an ovarian cDNA library using the Uni-ZAP cDNA library 30 construction system (Stratagene). The library was screened with the aforementioned human ZP2 cDNA probes to isolate additional cDNA clones that, together with those obtained with the RT-PCR, represented near full-length cDNAs. The nucleic acid sequence of these clones revealed 35 that the human ZP2 transcript has a single 2235 nt open reading frame that is 74% identical to that of the mouse ZP2 transcript. The human transcript encodes a 745 amino acid ZP2 protein with a calculated molecular mass of 82,356 Da that is 60.7% identical to that of the mouse ZP2

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protein (Liang et al., supra (1993)). The hydropathicity profiles of the human and mouse ZP2 proteins are remarkably similar and reflect the conserved nature of the allowable amino acid substitutions. Additional characteristics of this protein have been noted above.

These two examples demonstrate that the primary amino acid sequence of the zona pellucida proteins (ZP3, ZP2, ZP1) can be deduced from cloned zona genes (cDNAs and/or genomic clones). This data would not otherwise be available because the paucity of biological material makes impossible the direct determination of the zona protein sequences. Furthermore, this example demonstrates that the conservation of the zona genes among mammals permit the zona genes of one species (e.g. mouse) to be used to clone and characterize the zona genes of another species (e.g. human). Cross-hybridization data to genomic DNA from seven mammalian species further indicate that a similar strategy can be used to determine the primary protein structures of the zona proteins from any mammal.

Further, this invention provides cDNA and genomic clones that can be used to express recombinant zona proteins of mouse and human ZP2 and ZP3 in their entirety or in parts thereof. It will be obvious to those in the field that this can be done using a variety of viral or plasmid based vectors in a variety of prokaryotic and eukaryotic cell lines and in the production of transgenic animals. Such recombinant zona proteins may be of use as diagnostic reagents for the assessment of male fertility or lack thereof and for providing sufficient amounts of zona proteins for further biochemical characterization of structure-function correlates of the zona proteins.

Example 3: Identification of ZP3 and ZP2 Peptides Capable of Eliciting Antibodies that Bind to the Zona Pellucida Protein in the Same Species.

Prior to this invention, it had not been demonstrated that a peptide comprised of a portion of a zona protein from a particular species could elicit antibodies

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in that same species that would bind to the native zona pellucida structure and prevent fertilization. The success of demonstrating the efficacy of this approach is based on two aspects of this invention: the determination 5 of the primary amino acid sequence of the mouse and human ZP2 and ZP3 proteins by cloning the cognate genes (Ringuette et al., Dev. Biol. 127:287-95 (1988); Chamberlin et al., Dev. Biol. 131:207-14 (1989); Liang et 10 al., Mol. Cell. Biol. 10:1507-15 (1990); Chamberlin et al., Proc. Natl. Acad. Sci. USA 87:6014-18 (1990); Liang et al., Dev. Biol. 156:399-408 (1993)), and the identification of candidate regions on the zona proteins to test the efficacy of this contraceptive strategy. In addition, the determination that the zona pellucida proteins are 15 well conserved between mouse and human indicates that the three-dimensional structures of ZP2 and ZP3 in different mammalian species will have near identity. Thus, regions of the zona proteins identified as potential vaccine candidates in one species (e.g. mouse) will be effective 20 in other species (e.g. humans). These regions need not have identical amino acid sequence but need only be located in the homologous region of the zona pellucida matrix of each particular species.

As indicated above, once the primary amino acid 25 sequence of a protein is known, a variety of strategies can be used to identify candidate peptides for testing as contraceptive vaccines. An example of one strategy is provided in the invention.

The first candidate peptide was identified on 30 mouse ZP3 by screening an epitope expression library derived from a ZP3 cDNA with a monoclonal antibody specific to the ZP3 protein.

A 1.0 kb cDNA known to contain the epitope recognized by the anti-ZP3 monoclonal antibody (Ringuette 35 et al., supra (1986)) was cut into random fragments which were size selected (200 bp) and cloned into the λgt11 expression vector. More specifically, the cDNA insert of pZP3.1 was digested with DNase in the presence of 15 mM MgCl₂ and 200 bp size selected fragments (V. Mehra, D.

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Sweetser and R.A. Young, Proc. Natl. Acad. Sci. USA 83: 7013 (1986)) were ligated into Lambda ZAP (Strategene). *E. coli* BB4 cells were infected with the un-amplified epitope library and screened (Ringuelette et al., supra 5 (1986)), with an anti-ZP3 monoclonal antibody (East et al., Dev. Biol. 109: 268 (1985)). Positive clones were plaque purified and the sequence of the insert DNA was determined from isolated plasmid DNA (F. Sanger, S. Nicklen, et al., Proc. Natl. Acad. Sci. USA 74:5463 10 (1977)).

A synthetic peptide displaying an epitope for a contraceptive antibody. The nucleic acid sequence of the cDNA inserts from 8 positive clones was determined (FIG. 2A). The 24 nucleotides common to the eight clones code 15 for a seven amino acid peptide which must contain the epitope recognized by the antibody (FIG. 2B). The peptide represents amino acids 336-342 which is immediately adjacent to the most hydrophilic portion of ZP3 (FIG. 2C). A 16 amino acid peptide (ZP3 amino acids 328-343) containing the epitope (NH_2 -CYS-SER-ASN-SER-SER-SER-GLN-PHE-GLN-ILE-HIS-GLY-PRO-ARG-GLN-COOH) was synthesized (Merrifield, R.B., J. Amer. Soc., 85: 2149 (1963)) on a Model 430A, Applied Biosystems Solid Phase Synthesizer, deprotected and released from the phenylacetamidomethyl 25 resin with anhydrous hydrogen fluoride containing 10% anisole and 10% thiophenol at 0°C for 2 hr. The crude peptide was purified by HPLC on a Vydac C4 column and conjugated to keyhole limpet hemocyanin by coupling the amino terminal cysteine to KLH through a maleimido linkage 30 (Lerner, R.A. et al., Proc. Natl. Acad. Sci. USA, 78:3403 (1981)).

Immunogenicity of the synthetic peptide vaccine. Sixteen NIH random bred Swiss mice were immunized intraperitoneally with 100 µg of the ZP3 peptide-KLH 35 conjugate (1 mg/ml) in an equal volume of complete Freund's adjuvant and then boosted at 10-14 day intervals with 100 µg of conjugated peptide in incomplete Freund's adjuvant. Circulating anti-zona pellucida antibodies were detected using solubilized whole zona in an ELISA.

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Flexible ELISA plates were coated with purified, acid solubilized zona (J.D. Bleil and P.M. Wassarman, J. Cell Biol. 102:1363 (1986)) at 100 ng per well, blocked with 1% bovine serum albumin in Tris HCl, pH 7.5, 0.15 M NaCl 5 (TBS), and incubated with sera diluted 1:10⁴ in the same. The plates were washed several times with TBS/1% Tween-20, incubated with horse radish peroxidase (HRP) conjugated goat anti-mouse antibody, washed as before, and developed using a Horseradish Peroxidase Substrate Kit (Bio-Rad). 10 The response was quantified by measuring absorbance at 414nm.

A plateau level of the average response was reached after five immunizations. It should be noted that there was variation of the amount of circulating anti-zona 15 pellucida antibodies among the animals with the difference between the high and low responders being almost six-fold. Control animals were immunized with KLH alone using an identical regimen and had no detectable circulating anti-zona antibodies.

20 The reactivity of sera from immunized animals with individual zona proteins was analyzed using Western blots of purified zonae separated by SDS-PAGE. Isolated mouse zona were acid solubilized and separated by SDS-PAGE using 10% acrylamide (U.K. Laemmli, Nature 227:680 25 (1970)). Proteins were transferred to nitrocellulose (W.N. Burnette, Analyt. Biochem. 112:195 (1980)) and the filters soaked in TBS/1% BSA. Sera or antibodies were diluted in TBS/1% BSA/0.1% Tween and individual lanes were probed with: pre-immune sera diluted 1:50; immune sera 30 from KLH immunized mice diluted 1:50; immune sera from ZP3 peptide-KLH immunized mice diluted 1:50; rat anti-mouse ZP3 monoclonal antibody (East et al., Dev. Biol. 109:268-73 (1985)) diluted 1:50; and rabbit anti-mouse zona pellucida polyclonal antisera (East et al., supra (1985)) 35 diluted 1:50. Filters were washed in TBS/0.1% Tween and incubated with HRP-labeled second antibody of the appropriate specificity (Jackson Immunoresearch) diluted 1:1000 in TBS/BSA/Tween. Nitrocellulose-bound antibodies were visualized using 4-chloro-1-naphthol.

Sera from animals immunized with the ZP3 peptide-KLH conjugate reacted with a single zona protein which co-migrated with ZP3. No reaction with any of the zona proteins was detected with pre-immune or control sera.

To determine whether anti-peptide antibodies recognize zona in its native state as well as in acid-solubilized and SDS-denatured preparations, sera from experimental and control animals were used to stain unfixed frozen sections of mouse ovary. Ovaries were removed and immediately frozen in Tissue-Tek O.C.T. Compound (Lab-Tek Products) on dry ice. Five μm sections were mounted on gelatin coated slides, treated with 1% BSA in PBS for 15 min at 20°C and rinsed in PBS. Sections were treated for one hour with undiluted serum from immunized mice, rinsed in PBS and stained for 30 min at 20°C with FITC-conjugated goat anti-mouse IgG (Jackson ImunoResearch Laboratories) diluted 1:50 in PBS/BSA. Sections were rinsed with PBS, mounted in Fluormount-S (FisherBiotech) and photographed using Ektachrome 200 film.

Using a fluorescein-conjugated second antibody, mouse antibodies from experimental mice were detected binding to the zonae surrounding developing oocytes, indicating that the circulating anti-zona antibodies are capable of binding native ZP3 protein. There was no detectable fluorescence of sections stained with sera from control mice.

As evidence that alloimmunization with a zona pellucida peptide is not limited to the ZP3 protein, a second candidate peptide was identified on mouse ZP2 by screening an epitope expression library derived from a ZP2 cDNA with a monoclonal antibody specific to the ZP2 protein using the techniques described above. Specifically, a 0.9-kbp cDNA (pZP2.1) known to contain the epitope recognized by an anti-ZP2 monoclonal antibody (Liang et al., Mol. Cell. Biol. 10:1507-15 (1990)) was digested with DNase to create random fragments that were cloned into

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Lambda ZAP (Stratagene) to create an expression epitope library. The library was screened with the monoclonal antibody specific to mouse ZP2 and the nucleic acid sequence of positive clones was determined. The 54 bp common to the positive clones must encode the epitope recognized by the antibody. The peptide represents amino acids 114-129 which are coincident with the major hydrophilic portion of ZP2.

The 17 amino acid peptide (ZP2 amino acids 114-129) containing the epitope (NH_2 -Ile-Arg-Val-Gly-Asp-Thr-Thr-Asp-Val-Arg-Tyr-Lys-Asp-Asp-Met-COOH) was synthesized by Merrifield solid phase synthesis (see above) with an N-terminal cysteine with which it was coupled to keyhole limpet hemocyanin. Female mice immunized intraperitoneally with 100 μg of the ZP3 peptide-KLH conjugate (1 mg/ml) in equal volume of complete Freund's adjuvant and then boosted at 10-14 day intervals with 100 μg of conjugated peptide in incomplete Freund's adjuvant. Circulating anti-zona antibodies were detected in an ELISA as described above. After 6 immunizations, four of five female mice developed anti-zona antibodies at titers comparable to those immunized with the ZP3 peptide. These data demonstrate the ability to the ZP2 peptide to elicit antibodies that cross-react with zona pellucida from the same species

Example 4: A Contraceptive Vaccine Comprising a Synthetic Peptide with a ZP3 Epitope.

To determine if the circulating anti-ZP3 antibodies were of sufficient titer to bind to the zonae surrounding growing oocytes of the experimental mice, plastic embedded sections of ovaries isolated from four females immunized with ZP3-KLH conjugate were stained with horse radish peroxidase (HRP) conjugated anti-mouse antibody. Dissected ovaries were fixed for one hour in 1% glutaraldehyde, rinsed in PBS and embedded in JB4 plastic. Endogenous antibody was detected in 4 μm sections using an anti-mouse streptavidin-HRP kit (Zymed).

Mouse anti-zona pellucida antibodies were

observed coating the zonae of the oocytes in the sections examined. There were no detectable anti-zona antibodies in ovaries isolated from four control (KLH alone injected) mice. The ovarian sections of both the treated and 5 control animals contained only normal follicles and cell types with no evidence of inflammation or cellular cytotoxicity. The antisera of the ZP3-KLH immunized animals did not react with other mouse tissue including brain, liver, spleen, kidney, heart, lung, intestine, testis or 10 muscle (data not shown) which indicates that immunization with the peptide conjugate elicits a response that is specific for the zona pellucida.

Effectiveness of the synthetic peptide vaccine for contraception. The fertility of the remaining 12 15 experimental and 12 control mice was tested by mating them continuously with proven males. Two weeks after the last immunization, proven males were individually and continuously caged with experimental and control mice at a ratio of 1:1. The percentage of animals having given birth to 20 a litter versus the duration of continuous mating was compared for animals injected with ZP3 peptide-KLH and KLH alone. The titer of anti-ZP antibodies of three groups of ZP3 peptide-KLH immunized mice at the beginning of the mating period were averaged and, in order of increasing 25 average titers, were as follows: group 1, gave birth within 1 month (3 animals); group 2, gave birth between 4 and 7 months (3 animals); and group 3, did not give birth to litters within the 9 month study (6 animals).

In summary, all of the control (KLH alone 30 injected) mice gave birth to litters within three and a half weeks of the introduction of males. Three of the experimental, ZP3 peptide-KLH injected mice also gave birth within this period. These mice were among those that had the lowest titers (<0.2 A₄₁₄ units) of anti-zona 35 antibodies prior to mating. In the remainder of the experimental mice, a contraceptive effect was observed that lasted between 16 and 36 weeks at which time the study was terminated. Three of these animals gave birth to litters after 16 to 24 weeks and had intermediate anti-

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zona antibody titers. The remaining animals which remained infertile for the duration of the study had the highest initial titers and even 9 months after the last immunization had detectable circulating anti-zona antibodies.

The litter sizes of the ZP3-KLH treated animals which eventually became fertile ranged from 1-5 pups (average 2.8) whereas those treated with KLH alone had litters of 1-9 pups (average 5.2). Both groups had fewer than the normal 7-14 pups (average 10) which may be due, in part, to the adverse effects of intra-peritoneal administration of Freund's adjuvant on fecundity. In addition, the smaller litters of the KLH-ZP3 treated animals could be accounted for by the observed persistent low levels of circulating anti-zona antibodies some of which were detected binding to the zonae surrounding their intra-ovarian oocytes. Despite the presence of these low levels of anti-zona antibodies, these animals, when remated, gave birth to litters within three and a half weeks.

The foregoing invention has been described in some detail for purposes of clarity and understanding. It will also be obvious that various combinations in form and detail can be made without departing from the scope of the invention.

WHAT IS CLAIMED IS:

1. A contraceptive vaccine for use in a mammalian female comprising a polypeptide which consists
5 essentially of the mouse zona pellucida 2 (ZP2) amino acid sequence Ile-Arg-Val-Gly-Asp-Thr-Thr-Thr-Asp-Val-Arg-Tyr-Lys-Asp-Asp-Met or a portion thereof;

10 or a homologous mammalian amino acid sequence derived from a homologous region of a ZP2 protein or a portion of said homologous sequence, for binding of an antibody that inhibits fertilization of an egg by a sperm;

15 said mammalian amino acid sequence being included in a zona pellucida protein originating from the species in which said vaccine is used; and

15 said vaccine further comprising a pharmacologically acceptable vehicle.

2. The contraceptive vaccine of claim 1, wherein said homologous mammalian amino acid sequence is
20 Ile-Arg-Val-Met-Asn-Asn-Ser-Ala-Ala-Leu-Arg-His-Gly-Ala-Val-Met and said sequence is derived from a human ZP2 protein.

3. The contraceptive vaccine according to
25 claims 1 or 2, wherein said mammalian female in which said vaccine is used is selected from the group consisting of: a cat, a dog, a pig, a cow, and a woman.

4. The contraceptive vaccine according to
30 claims 1, 2, or 3, further comprising an effective amount of an adjuvant.

35 5. A contraceptive vaccine comprising a polypeptide which consists essentially of a synthetic peptide corresponding to the mouse zona pellucida (ZP2) amino acid sequence Ile-Arg-Val-Gly-Asp-Thr-Thr-Thr-Asp-Val-Arg-Tyr-Lys-Asp-Asp-Met or a portion thereof;

or a synthetic peptide corresponding to a homologous mammalian amino acid sequence derived from a

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homologous region of a ZP2 protein or a portion of said homologous sequence,

for binding of an antibody that inhibits fertilization of an egg by a sperm;

5 said mammalian amino acid sequence being included in a zona pellucida protein originating from the species in which said vaccine is used; and

said vaccine further comprising a pharmacologically acceptable vehicle.

10

6. The contraceptive vaccine of claim 5, wherein said homologous mammalian amino acid sequence is Ile-Arg-Val-Met-Asn-Asn-Ser-Ala-Ala-Leu-Arg-His-Gly-Ala-Val-Met and said sequence is derived from a human ZP2 15 protein.

7. The contraceptive vaccine according to claims 5 or 6, wherein said mammalian female in which said vaccine is used is selected from the group consisting of: 20 a cat, a dog, a pig, a cow, and a woman.

8. The contraceptive vaccine according to claims 5, 6, or 7, further comprising an effective amount of an adjuvant.

25

9. A polypeptide composition consisting essentially of the mouse zona pellucida 2 (ZP2) amino acid sequence Ile-Arg-Val-Gly-Asp-Thr-Thr-Thr-Asp-Val-Arg-Tyr-Lys-Asp-Asp-Met or a portion thereof;

30 or a homologous mammalian amino acid sequence derived from a homologous region of a ZP2 protein or a portion of said homologous sequence.

10. The polypeptide composition of claim 9, 35 wherein said homologous mammalian amino acid sequence is Ile-Arg-Val-Met-Asn-Asn-Ser-Ala-Ala-Leu-Arg-His-Gly-Ala-Val-Met and said sequence is derived from a human ZP2 protein.

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11. The polypeptide composition of claim 9 or
10, bound to a conjugate.

12. An antibody which binds to the polypeptide
5 composition of claim 11.

FIGURE 1(A)

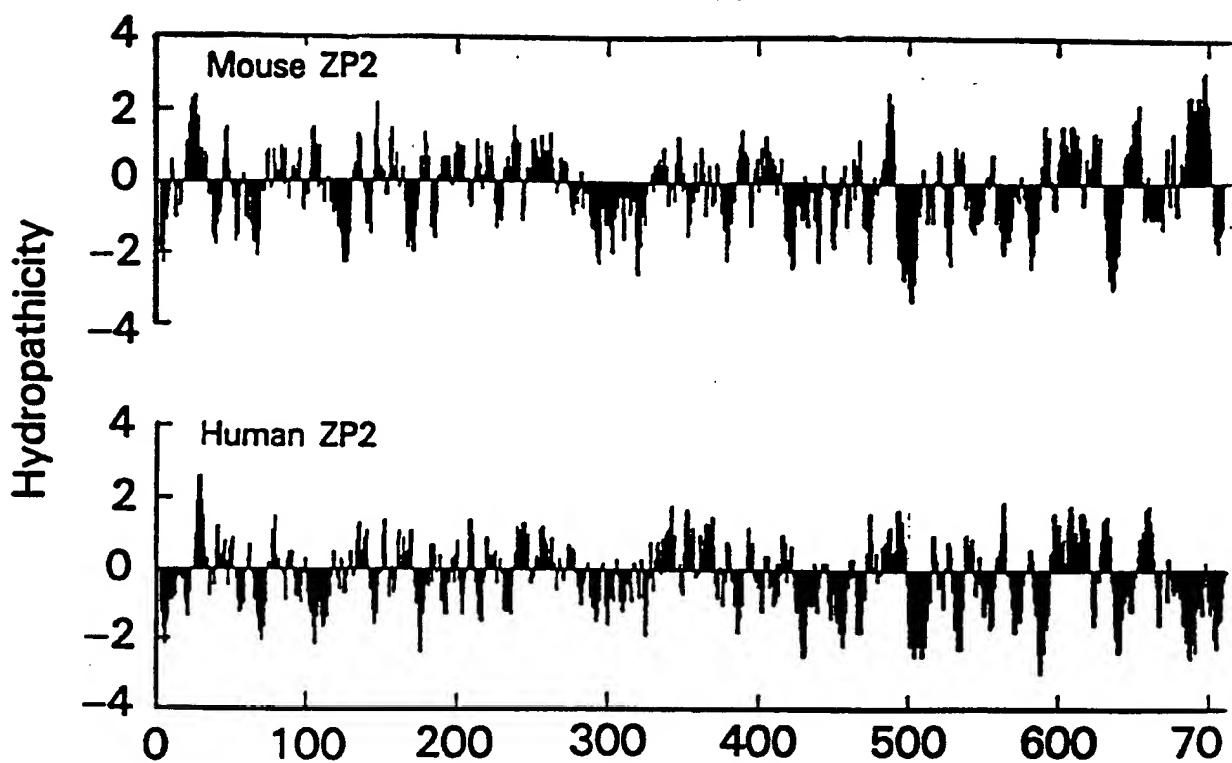


FIGURE 1(B)

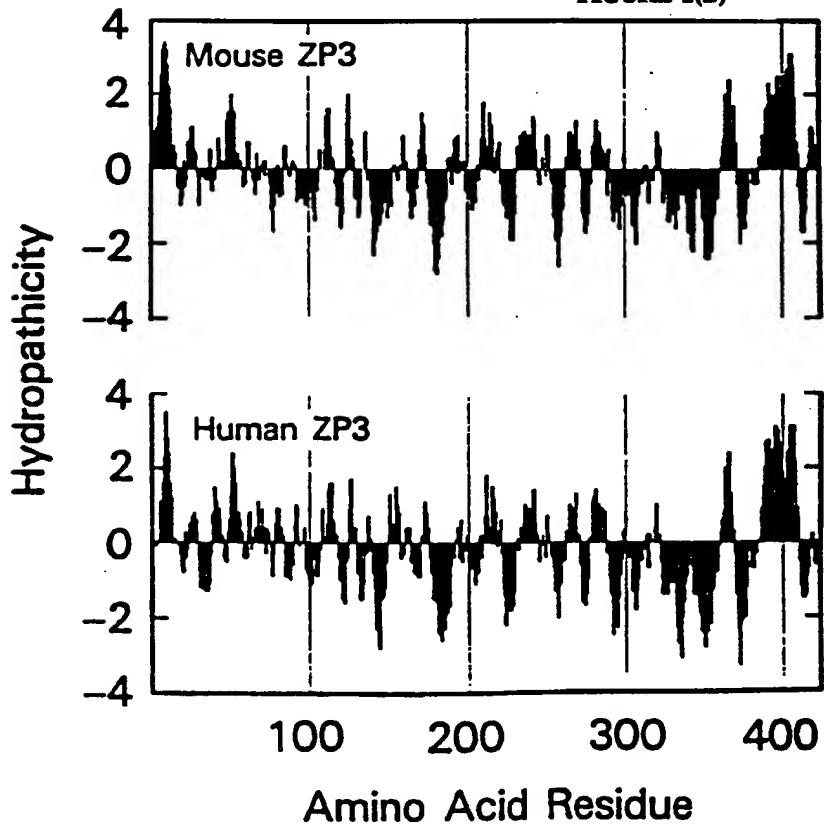


FIGURE 2(A)

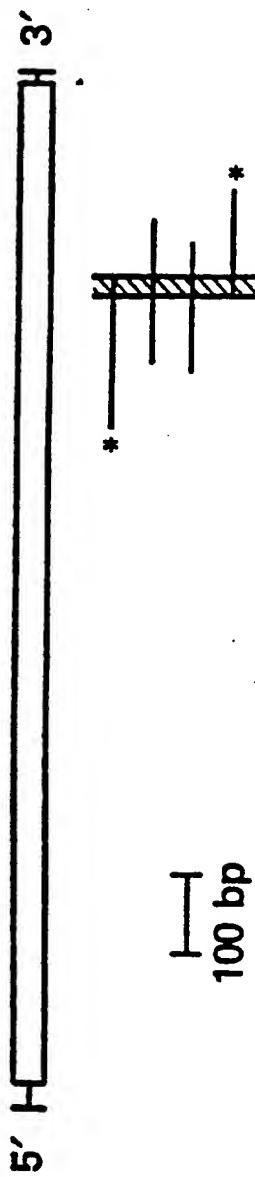
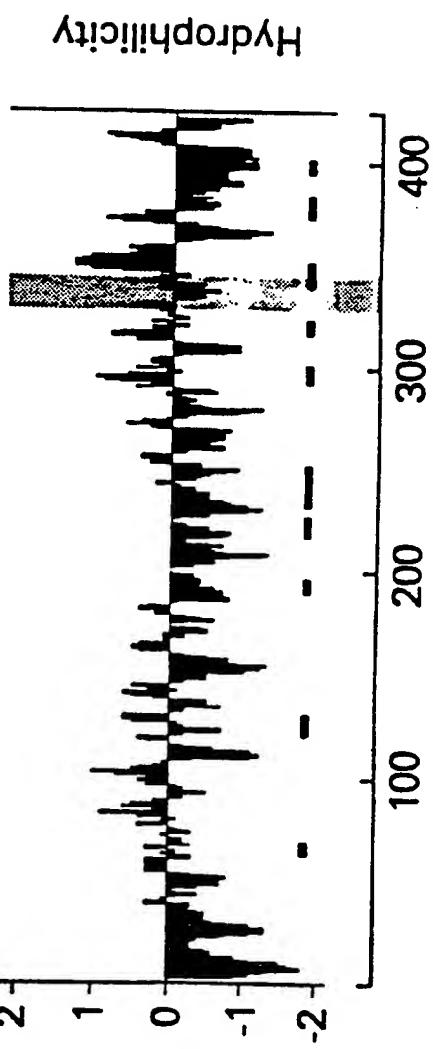


FIGURE 2(B)

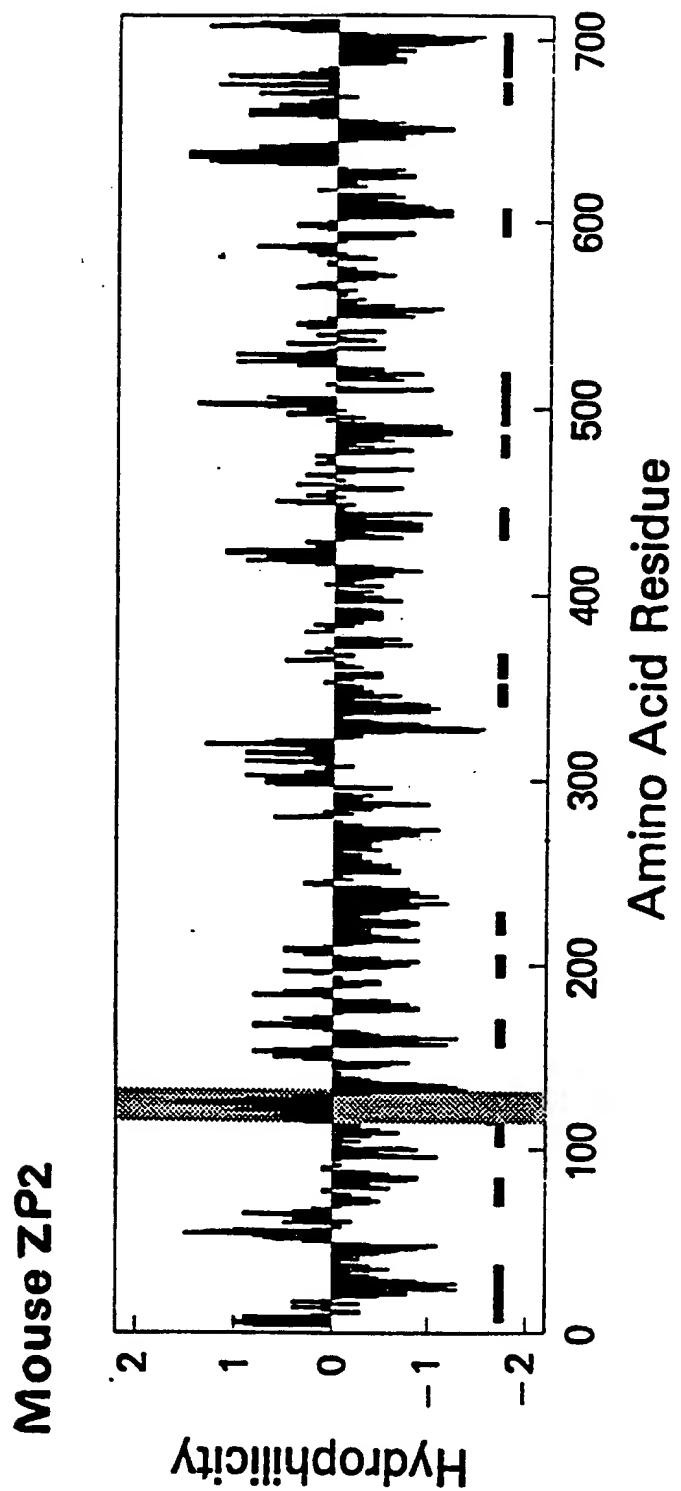
AG TTC CAG ATC CAT GGA CCC CGC C
 NH_2 -cys ser asn ser ser ser gln PHE GLN ILE HIS GLY PRO ARG gln-cooh

FIGURE 2(C)



3/5

FIGURE 3(A)



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FIGURE 3(B)

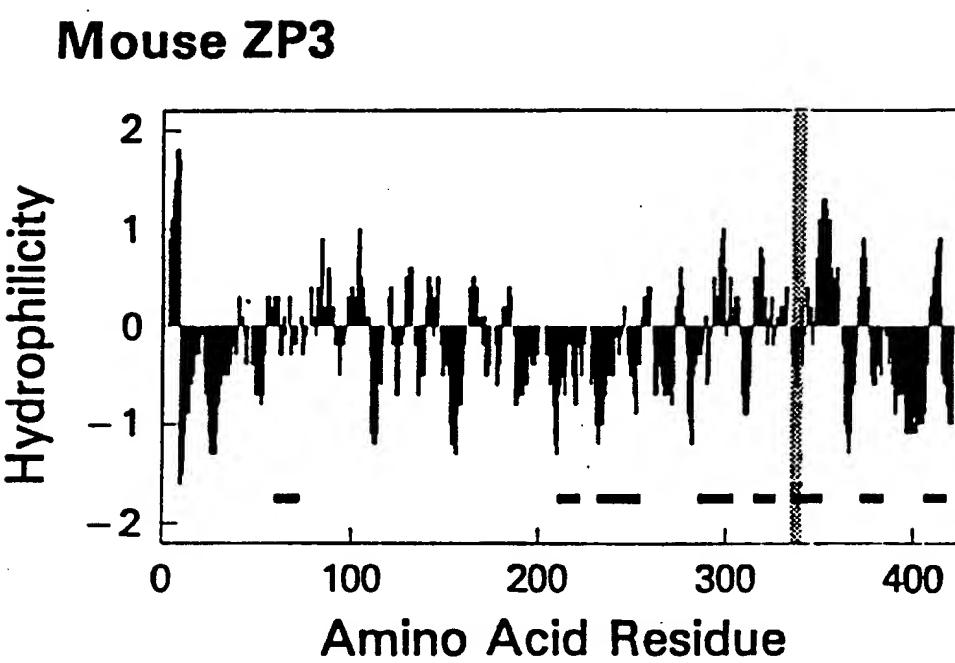


FIGURE 4(A)

ZP3 Epitope

328	$\text{NH}_2\text{-Cys-Ser-Asn-Ser-Ser-Ser-Gln-Phe-Gln-Ile-His-Gly-Pro-Arg-Gln-COOH}$	343
327	$\text{NH}_2\text{-Cys-Gly-Thr-Pro-Ser-His-Ser-Arg-Gln-Pro-His-Val-Met-Ser-Gln-COOH}$	
327		342

FIGURE 4(B)

292 Epitope

	mouse	human	114	118	129	133
NH ₂ -Ile-Arg-val-Gly-Asp-Thr-Thr-Asp-Val-Arg-Tyr-Lys-Asp-Met-COOH						
NH ₂ -Ile-Arg-Val-Met-Asn-Ser-Ala-Ala-Leu-Arg-His-Gly-Ala-Val-Met-COOH						

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/03289

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 5 A61K39/00 C07K7/08 A61K39/385 C07K15/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 5 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,90 15624 (THE UNITED STATES OF AMERICA) 27 December 1990 see the whole document ---	1-12
A	WO,A,92 03548 (AKZO) 5 March 1992 see the whole document ---	1-12
A	MOLECULAR AND CELLULAR BIOLOGY vol. 10, no. 4 , April 1990 , WASHINGTON US pages 1507 - 1515 LIANG L. ET AL. 'Oocyte-Specific Expression of Mouse Zp-2: Developmental Regulation of the Zona Pellucida Genes' cited in the application see the whole document ---	1-12
		-/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

25 August 1994

Date of mailing of the international search report

31-08-1994

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Moreau, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/03289

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SCIENCE vol. 246 , 17 November 1989 , LANCASTER, PA US pages 935 - 938 MILLAR ET AL. 'Vaccination with a Synthetic Zona Pllucida Peptide produces Long-Term Contraception in Female Mice' see the whole document -----</p>	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/03289

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		AU-A-	5826790	08-01-91
		CA-A-	2058999	13-12-90
		EP-A-	0477226	01-04-92
		JP-T-	5500654	12-02-93
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WO-A-9203548	05-03-92	AU-A-	8328591	17-03-92
		CA-A-	2090486	28-02-92
		CN-A-	1060499	22-04-92
		HU-A-	64394	28-12-93
		JP-T-	6500690	27-01-94
		NZ-A-	239518	25-03-94
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